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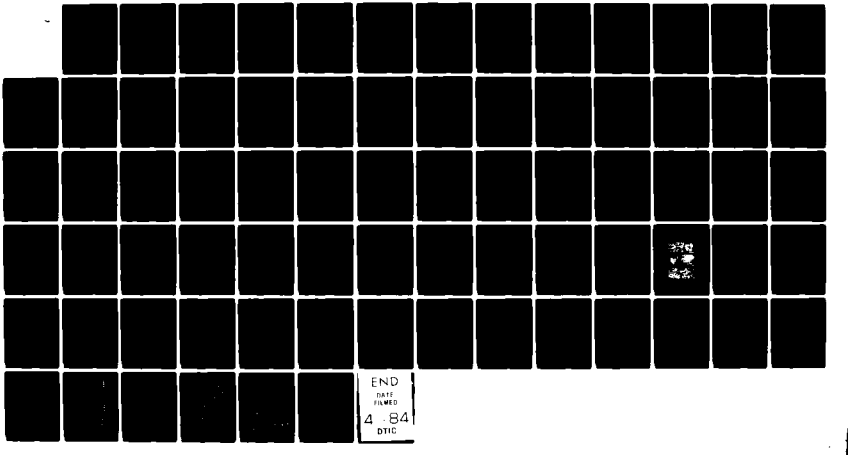

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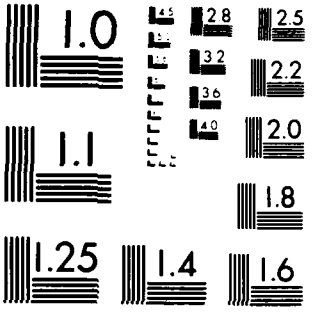

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Interim Technical Report

CHEMICAL CARCINOGEN-INDUCED CHANGES IN tRNA METABOLISM IN HUMAN CELLS

Ronald W. Trewyn
Department of Physiological Chemistry

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It is hypothesized that transfer RNAs mediate endogenous promotion of carcinogenesis subsequent to chemical carcinogen initiation, and that without the appropriate changes in tRNA metabolism, the ultimate expression of the neoplastic state will not be attained. Current studies are concentrating on tRNA ribosyltransferase modification reactions which are considered to be the pivotal molecular aberrations in this process. A normal human cell culture model system responsive to phorbol ester tumor promoters was developed which allows the evaluation of the role of tRNA in promotion of carcinogenesis. Chronic exposure to the tumor promoters induces a transient 5 to 10-fold increase in the saturation density of human cells if the treatment is initiated at early population doublings in culture in medium supplemented with elevated levels of specific amino acids. A significant decrease in queuine modification in the anticodon of cellular tRNAs precedes the transient 5 to 10-fold increase in saturation density, and queuine modification increases prior to —

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18. modifications, queuosine, inosine, tRNA ribosyltransferases, queuine, hypoxanthine.

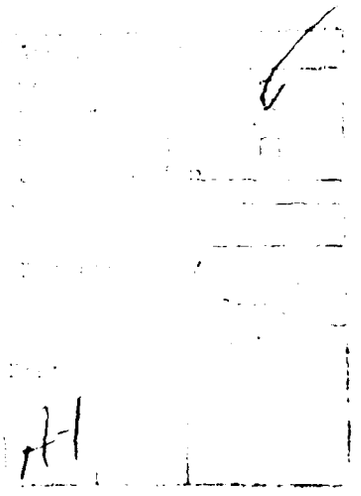
19. the subsequent decrease in saturation density. The increase in queuine modification correlates to the induction of an endogenous queuine salvage pathway. Most importantly, the addition of excess exogenous queuine inhibits the transient increase in saturation density induced by the tumor promoters, i.e., it blocks the expression of a transformed phenotype.

Recent studies have also led to the identification of an enzyme which incorporates hypoxanthine into mature tRNA macromolecules. The previously undocumented hypoxanthine incorporating activity has been assayed in rat liver and human leukemia cell cytosolic preparations, and it was resolved from the queuine incorporating enzyme (tRNA-guanine ribosyltransferase) by DEAE-cellulose column chromatography. The enzyme assay developed is based on the incorporation of radiolabelled hypoxanthine into unfractionated tRNA from various sources, and the reaction rate is proportional to the amount of added enzyme extract. Hydrolysis of the radiolabelled tRNA and analysis of the nucleoside composition yields inosine (the nucleoside of hypoxanthine) as the only radiolabelled product. It is proposed that the enzyme, a tRNA-hypoxanthine ribosyltransferase, is responsible for the biosynthesis of inosine in the anticodon wobble position of specific tRNAs, resulting in greatly expanded codon recognition by these tRNAs. It is further proposed that altered queuine and hypoxanthine modification in the anticodons of their respective tRNAs is involved in the aberrant gene expression associated with carcinogenesis. Blocking these structural alterations in tRNA might thus block the expression of carcinogenesis.

Chemical Carcinogen-Induced Changes in tRNA Metabolism in Human Cells

Ronald W. Trewyn, Ph.D.
Associate Professor of Physiological Chemistry

Interim Report (10/1/82 to 9/30/83)
AFOSR-80-0283



B. Research Objectives

The grant proposal submitted in 1980 tendered the hypothesis that changes in tRNA metabolism are required for the progression of cells through the stages of carcinogenesis. Following the initiation of the carcinogenic process, gradual phenotypic modulation towards a less differentiated cellular state occurs. Alterations in tRNA modification and catabolism are known to commence soon after chemical carcinogen exposure, and the accruing changes in tRNA isoaccepting species may be involved in releasing the post-transcriptional controls over developmental gene expression.

At some early time after the initiation of carcinogenesis there is an elevation of RNA methylation which likely leads to the increase in the levels of methylated RNA catabolites. These catabolites may modulate tRNA modification further, either by inhibiting anticodon modifications by tRNA ribosyltransferases or by acting as alternate substrates for these enzymes. New tRNA isoaccepting species are generated by the changes in macromolecular modification and these species should translate different (e.g. fetal) mRNAs that are present. By this method, onco-developmental proteins would be synthesized. Some of the new proteins could be tRNA methyltransferases, and this would start the cycle over again at a more aberrant level. After many such cycles, the stage of neoplastic transformation could be reached. We are examining the variations in RNA metabolism to determine if they are, in fact, involved in the dedifferentiation and progression of carcinogenesis.

C. Status of the Research

We discovered previously that the mouse skin tumor promoter phorbol 12,13-didecanoate (PDD) will promote significant increases in the saturation density of normal human cells cultured in medium containing 8X nonessential amino acids [Trewyn and Gatz, in press (appended)]. The saturation density for the normal control cells was approximately 4×10^4 cells/cm^2 at most passages, while the PDD treated cells maintained levels 3 to 4-fold higher for most of the lifetime of the cultures. The specificity of the effect for an active tumor promoter was demonstrated by the fact that the inactive analog 4α-phorbol 12,13-didecanoate (4α-PDD) gave results similar to the untreated controls. Because aspartic acid and asparagine (2 of the 4 amino acids normally using queuine modified tRNAs) are nonessential amino acids, we examined combinations of amino acids which include all 4 of those for queuine tRNAs. We discovered an even greater initial increase in the saturation density of normal cells when they were treated with PDD in medium supplemented with 2X aspartic acid, asparagine, histidine, tyrosine (the queuine tRNAs; some of which are hypomodified in transformed cells) and phenylalanine (its tRNA is usually hypomodified for wye base in transformed cells). Cell densities in the PDD treated cultures reached levels 5 to 10-times those in control cultures. Although the timing of this transient increase in density has been somewhat variable, all the cultures exhibited a sustained 2 to 4-fold elevation subsequently, similar to that seen in 8X nonessential amino acids. Removal of PDD from the culture medium resulted in a return to near-normal saturation density within a few population doublings. The PDD treated cultures remained in exponential growth at cell densities greater than 10-fold higher than the control cultures. Anchorage-independent growth of normal human cells was also promoted by PDD in a dose-dependent manner, with prior subculturing in the presence of PDD being required for maximal colony formation [Trewyn and Gatz, in press (appended)]. The phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate alone or in combination with other co-promoters also induced phenotypic changes in normal human cells under the prescribed culture conditions [Davakis and Trewyn, 1983 (appended)]. These reversible phenotypic changes are characteristic of mimicry of transformation as induced in rodent cells by the phorbol esters.

As described previously [Elliott and Trewyn, 1982; Trewyn et al., 1983 (appended)], 7-methylguanine induces queuine hypomodification of tRNA and promotes the expression of transformed phenotypes in normal Chinese hamster cells. We now have evidence that phorbol ester tumor promoters elicit similar changes in normal human cells (Elliott et al., in preparation). Significant increases in queuine hypomodification of tRNA were observed in human cells treated continuously with PDD, and this increase preceded the transient increase in saturation density promoted by PDD. The timing of both the increase in queuine hypomodification and saturation density have been somewhat variable from one primary culture to another, but in five independent experiments, maximal queuine hypomodification preceded the maximal increase in saturation density. In all experiments, a decrease in queuine hypomodification then preceded a decrease in saturation density. The inactive tumor promoter 4α-PDD had a minimal effect on queuine hypomodification and saturation density.

Based on the known molecular mechanism for queuine modification (a base exchange for guanine catalyzed by tRNA-guanine ribosyltransferase), the transient nature of the hypomodification induced in the continuous presence of PDD was unexpected. However, it was discovered that newly established cultures of human skin fibroblasts lack the ability to salvage endogenous queuine from tRNA turnover, whereas later passage cells aquire this ability (Elliott et al., in preparation). When first passage human skin cells were radiolabelled with [^{3}H]dihydroqueuine, the presence or absense of exogenous queuine had no effect on the half-life of the acid insoluble radioactivity, thereby indicating the lack of queuine salvage in these cells. However, at the fourth passage of cells derived from the same primary culture, the half-life in queuine-free medium was appreciably longer (>20 days) than that in queuine-containing medium (1.5 days), indicating the induction of the queuine salvage pathway. Again, the timing of induction of maximal queuine salvage from endogenous tRNA turnover was somewhat variable from one primary culture to another, but it was usually attained by 15 to 20 population doublings (4 to 6 passages) in culture.

The serum utilized to supplement the cell culture medium contains queuine, and that is the source of queuine for tRNA modification in cultured cells. Our data indicated, however, that a PDD-induced, queuine limitation in the cells had some role in the increase in saturation density. Therefore, excess queuine (5×10^{-8} M) was added to cells treated with PDD (10^{-8} M), and saturation densities were monitored. The excess queuine significantly reduced the increase in saturation density (from 5-fold to 2-fold) induced by PDD, while having little effect on saturation densities in the absence of PDD (Elliott et al., in preparation). These data indicate that queuine hypomodification of tRNA can mediate the expression of transformed phenotypes; an observation consistent with our original hypothesis.

Investigations into tRNA modifications within the anticodon region have led to the identification of an enzymatic activity which incorporates hypoxanthine into mature tRNA macromolecules [Elliott and Trewyn, in press (appended)]. This enzyme is postulated to be similar to tRNA-guanine ribosyltransferase which inserts queuine into the wobble base position of certain tRNAs generating the nucleoside queuosine. The previously undocumented hypoxanthine incorporating enzyme has been assayed in both rat liver and human leukemia cell cytosolic preparations, and it has been resolved from tRNA-guanine ribosyltransferase by DEAE-cellulose column chromatography. Whereas tRNA-guanine ribosyltransferase has substrate preferences for guanine and yeast tRNA in vitro, the tRNA-hypoxanthine ribosyltransferase utilizes hypoxanthine and E. coli tRNA much more readily. The vast differences in substrate utilization and in column elution characteristics indicate that unique enzyme activities are being assayed.

The tRNA-hypoxanthine ribosyltransferase assay is based on the incorporation of radiolabelled hypoxanthine into unfractionated tRNA. The reaction rate is proportional to the amount of substrate E. coli tRNA, as well as the amount of added enzyme extract. Most importantly, subsequent to the in vitro assay, radiolabelled inosine (the nucleoside of hypoxanthine) is the predominant product found in tRNA hydrolysates. It is proposed that the enzyme, a tRNA-hypoxanthine ribosyltransferase, is responsible for the normal biosynthesis of inosine in the anticodon wobble position of specific tRNAs, resulting in greatly expanded codon recognition by these tRNAs. This enzyme could be of profound importance in regulating gene expression at the translational level, and therefore, it may be of great significance to the hypothesized role for tRNA in carcinogenesis. Consistent with this possibility are recent data demonstrating a reversal of the PDD-induced increase in the saturation density of human cells by hypoxanthine. Therefore, both substrates for cellular tRNA ribosyltransferases, queuine and hypoxanthine, block the expression of a transformed phenotype induced by a known tumor promoter. The ability to modulate these tRNA anticodon modifications might thus allow one to block carcinogenesis subsequent to the initiation event.

D. Publications

Trewyn, R.W., Elliott, M.S., Glaser, R., and Grever, M.R. Alterations in tRNA metabolism as markers of neoplastic transformation. In: Biochemical and Biological Markers of Neoplastic Transformation (P. Chandra, ed.), pp. 263-276, Plenum Publishing Corp., New York, 1983.

Davakis, L.A. and Trewyn, R.W. Evaluating tumor promoter activity in vitro with human diploid fibroblasts. In: Polynuclear Aromatic Hydrocarbons: Formation, Metabolism, and Measurement (M. Cooke and A.J. Dennis, eds.), pp. 393-404, Battelle Press, Columbus, 1983.

Trewyn, R.W. and Gatz, H.B. Altered growth properties of normal human cells induced by phorbol 12,13-didecanoate. In Vitro (in press).

Elliott, M.S. and Trewyn, R.W. Inosine biosynthesis in transfer RNA by an enzymatic insertion of hypoxanthine. J. Biol. Chem. (in press).

Elliott, M.S., Katze, J.R., and Trewyn, R.W. A tumor promoter-induced decrease in queuine modification of tRNA in normal human cells correlates to the expression of an altered cell phenotype. Cancer Res. (in preparation).

E. Personnel

Ronald W. Trewyn, Ph.D., Associate Professor of Physiological Chemistry, Principal Investigator.

Eric D. Utz, Technician

Dawn E. Gibboney, Technician.

Lani A. Davakis, M.S., 1983.
Thesis: Reponse of Human cells to Tumor Promoters.

Mark S. Elliott, Ph.D., 1983.
Thesis: Biosynthesis of Queuosine and Inosine in the First Position of the Anticodon in Transfer RNA: Implications in Neoplasia.

Keith A. Kretz, Ph.D. Candidate.

F. Interactions

American Society of Biological Chemists, San Francisco, California, June 5-9, 1983.

Presentation: Enzymatic insertion of hypoxanthine into transfer RNA.

Published Abstract: Elliott, M.S. and Trewyn, R.W. Fed. Proc. 42: 2149, 1983.

Review of Air Force Sponsored Basic Research in Biomedical Sciences, Irvine, California, July 26-28, 1983.

Presentation: Transfer RNA-mediated endogenous promotion of carcinogenesis.

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NEOPLASTIC TRANSFORMATION
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ALTERATIONS IN tRNA METABOLISM AS MARKERS OF NEOPLASTIC TRANSFORMATION

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INTRODUCTION

Numerous changes in tRNA modification and catabolism are observed when cells undergo neoplastic transformation. The nature of certain of these changes is well established, while others require further characterization. Enzymes involved in the macromolecular modification of tRNA, the tRNA methyltransferases, exhibit idiosyncratic alterations during neoplastic transformation. These alterations include increases in enzyme specific activity as well as the appearance of different tRNA methyltransferases in the malignant tissue[1,2]. The increased tRNA methyltransferase activity and capacity observed *in vitro* for the enzymes isolated from transformed cells can also be correlated to increased methylation of specific tRNA isoaccepting species, although not total tRNA, *in vivo*[3].

Elevated turnover of tRNA is another characteristic of neoplastic transformation[4,5], and the increased rate of tRNA catabolism may explain the lack of extensive hypermethylation of total tRNA in malignant cells. Most modified nucleosides in tRNA cannot be salvaged when the macromolecules are degraded, and therefore, they are excreted[4]. Monitoring the elevated excretion of these tRNA catabolites by cancer patients is being investigated to determine the usefulness of these components as biochemical markers for cancer[6-8].

The enhanced generation of methylated tRNA catabolites by cancer patients may also have a fundamental role in the neoplastic process. Chronic exposure of normal mammalian cells in culture to specific methylated purine RNA catabolites can lead to neoplastic transformation[9,10]. However, the mode of action by which these natural products elicit such a response has not been established.

The appearance of many unique tRNA isoaccepting species is another common feature established for malignant cells[4,11,12]. In some cases, these species appear to differ from their normal counterparts with respect to their macromolecular modifications. Hypomodification for Y-base adjacent to the anticodon in phenylalanine tRNA and for Q-base in the first position of the anticodons for histidine, tyrosine, asparagine, or aspartic acid tRNA's is responsible for the appearance of some of the different isoacceptors in transformed cells[12-14]. Again, these tRNA aberrations offer biochemical markers for neoplastic transformation.

In this report, we examine certain of the alterations in tRNA metabolism associated with neoplasia. Potential interrelationships between the changes in tRNA modification and catabolism are explored, and a role for these aberrations in the expression of carcinogenesis is postulated.

MATERIALS AND METHODS

Nucleosides in urine were resolved and quantitated using reversed-phase high performance liquid chromatography[8] following clarification on a boronate column[15]. Quantitation was relative to the creatinine content in random urine specimens[7].

Establishment and propagation of primary cultures of Chinese hamster embryo cells were as previously described[9,16] except that the culture medium was supplemented with only 5% fetal bovine serum. These cells typically exhibit a finite lifetime in culture of 10 to 12 passages under the conditions employed. The methods for transforming these cells by chronic exposure to selected methylated purines have also been published[9,10]. The concentration of methylated purine utilized was always 10 μM.

The assay for Q-deficient tRNA makes use of the enzyme tRNA transglycosylase from Escherichia coli[14]. This enzyme can utilize mammalian tRNA's for histidine, tyrosine, asparagine, and aspartic acid as substrates only if they are hypomodified, i.e., the tRNA's have guanine in the first position of the anticodon instead of Q-base[14]. Transfer RNA from proliferating Chinese hamster cells treated with 10 μM 7-methylguanine was isolated utilizing published protocols[17], and it was evaluated as a substrate for the E. coli enzyme. The assay for Q-hypomodification involves an exchange

reaction with [8-^{3}H]guanine. Previously published methods were employed[14,18]. Yeast tRNA is Q-deficient, and therefore, was utilized as a positive control. E. coli tRNA is Q-sufficient, so it was used as a negative control. A tRNA transglycosylase from rabbit erythrocytes[19] was used to assess enzyme inhibition by 7-methylguanine.

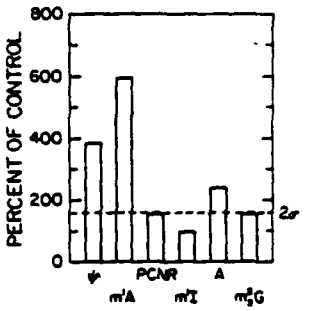

Fig. 1. Excretion of nucleoside markers by a patient at the time of of diagnosis of NPC. The results were calculated as nmoles nucleoside/μmole creatinine, and are expressed relative to normal values as percent of control. The dashed line denotes the position of two standard deviations above normal for adenosine, the marker exhibiting the largest relative standard deviation. Excretion levels above the dashed line represent significant ($P<0.02$) increases for adenosine and highly significant ($P<0.01$) for the other nucleosides. The abbreviations are: ψ, pseudouridine; m^1A, 1-methyladenosine; PCNR, 2-pyridone-5-carboxamide-N'-ribofuranoside; m^1I, 1-methylinosine; A, adenosine; and m_2^2G, N^2,N^2-dimethylguanosine.

RESULTS

Nucleoside Excretion by Cancer Patients

Urinary nucleoside excretion has been quantitated for patients with nasopharyngeal carcinoma (NPC) and leukemia, and the results

have been compared to normal excretion levels. The normal excretion values (nmoles nucleoside/μmole creatinine) used for comparison were as follows[8]: pseudouridine, 24.8; 1-methyladenosine, 2.02; 2-pyridone-5-carboxamide-N'-ribofuranoside, 1.14; 1-methylinosine, 0.96; 1-methylguanosine, 0.70; adenosine, 0.23; and N^2,N^2-dimethylguanosine, 1.05. The greatest relative standard deviation for the controls was 30.4% for adenosine.

The relative nucleoside excretion pattern for a Caucasian NPC patient at the time of diagnosis is presented in Fig. 1. At that time, the excretion levels of pseudouridine and 1-methyladenosine

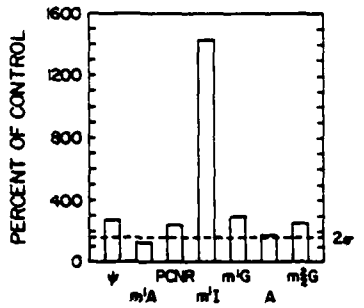

Fig. 2. Excretion of nucleoside markers by a patient at the time of diagnosis of AML. See legend to Fig. 1 for details. The additional abbreviation is: m^1G, 1-methylguanosine.

were elevated 4-fold and 6-fold respectively. Cells from tumor tissue biopsies contained the Epstein-Barr virus (EBV) genome (12.5 equivalents/cell)[20], and the patient's serum contained very high levels of antibodies to an EBV-specific DNase (10.2 units neutralized/ml serum).

The nucleoside excretion pattern for an individual at the time of diagnosis of acute myelogenous leukemia (AML) is shown in Fig. 2. In this case, 1-methylinosine was the primary marker with an increase of greater than 14-fold. Four of the other nucleosides (pseudouridine, 2-pyridone-5-carboxamide-N'-ribofuranoside, 1-methylguanosine, and N^2,N^2-dimethylguanosine) were elevated

approximately 3-fold. This patient also had significantly elevated adenosine deaminase levels in his peripheral blood cells (21.0 units/10^6 cells) compared to normal (8.4 units/10^6 cells).

Cell Transformation by Methylated Purines

The significant increase in the excretion of modified RNA catabolites by cancer patients led to the examination of the response of normal mammalian cells to these components[9,10]. Certain methylated purines were found to transform Chinese hamster embryo cells *in vitro*, with neoplastic transformation being demonstrated in some cases[10]. A summary of methylated purines evaluated and those transforming the cells for proliferative capacity (finite to continuous lifetime in culture) can be seen in Table 1. Certain of the RNA catabolites (1-methylguanine and 7-methylguanine) greatly enhance the generation of continuous cell lines, while others do not. Other naturally occurring methylated purines (7-methylxanthine and 1,3,7-trimethylxanthine) are also quite effective in transforming the cells.

The expression of various transformed phenotypes appearing during continuous exposure to the methylated purines can be reversed by removal of the methylated purine. An example showing increased saturation density of a 7-methylxanthine-transformed cell line is presented in Fig. 3. Removal of 7-methylxanthine at passage level 15, a passage level exceeding the normal number of passages obtainable before senescence and cell death, resulted in a significant decrease in the cell density of subsequent passage levels. In 3 of 4 independent transformation experiments with 7-methylxanthine, the cultures went through such a "crisis"

Table 1. Methylated Purines Generating Continuous Chinese Hamster Cell Lines

Methylated Purine	Continuous/Treated
None (Control)	2/30
Guanine (Control)	0/2
1-Methyladenine	0/2
1-Methylguanine	15/16
3-Methylguanine	0/2
7-Methylguanine	11/12
1-Methylhypoxanthine	0/2
1-Methylxanthine	1/4
3-Methylxanthine	0/2
7-Methylxanthine	4/4
1,3,7-Trimethylxanthine	4/6

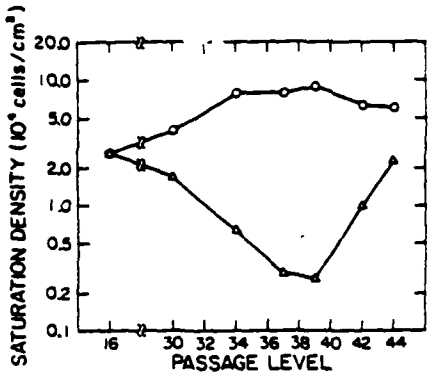

Fig. 3. Saturation densities for a 7-methylxanthine-transformed Chinese hamster cell line. The cells had been treated continuously with 10 μM 7-methylxanthine since the first passage of the primary culture. When the transformed, "continuous" cell line was subcultured at passage 15, duplicate cultures were maintained thereafter in the presence (o) or absence (Δ) of 7-methylxanthine. Saturation densities were determined after confluent cultures were split 1:4 and allowed to grow for 7 days. Duplicate cultures were trypsinized, and cells were counted with a hemacytometer.

period after removal of the methylated purine. With a 1,3,7-trimethylxanthine-transformed cell line, the cloning efficiency in soft agar decreased 6-fold after removal of the methylxanthine (unpublished observation), and tumorigenicity in nude mice was reversed by removal of 1-methylguanine from a corresponding cell line[21]. In the latter case, there was no change in the cloning efficiency in soft agar or any other in vitro characteristic related to transformation.

Q-Hypomodification of Cellular tRNA

The enzyme tRNA transglycosylase from mammalian sources catalyzes the reaction depicted in Fig. 4. Transfer RNA isolated from normal cells is mainly in the Q-modified form, while tRNA from transformed cells is Q-deficient[14]. The possibility that 7-methylguanine, a structural analog of Q-base, might inhibit Q-modification of tRNA was examined by treating normal Chinese hamster cells with 10 μM 7-methylguanine; the same concentration and conditions used for transformation. Transfer RNA was isolated from treated and untreated normal cells after 6 population doublings and assayed for Q-deficiency. Transfer RNA was also isolated from cells treated for 4 population doublings followed by no treatment for 2 more doublings to assess reversibility of any 7-methylguanine-induced Q-hypomodification. As can be seen in Table 2, 7-methylguanine induced Q-hypomodification of tRNA in the cells, and the Q-deficiency was reversible.

A tRNA transglycosylase isolated from rabbit erythrocytes was also shown to be inhibited by 10 μM 7-methylguanine *in vitro*. In 4 separate experiments with Q-deficient yeast tRNA, the percent inhibition obtained was 60.1 ± 7.6 (mean ± standard deviation) when the guanine substrate concentration was 1 μM.

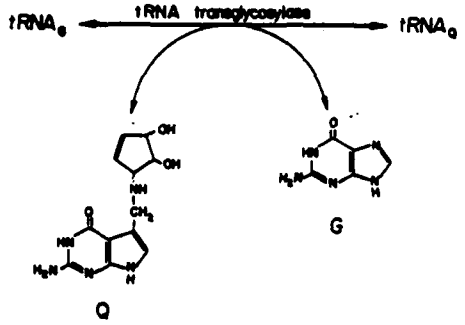

Fig. 4. tRNA transglycosylase reaction responsible for exchanging Q-base for guanine in the first position of the anticodon of tRNA's for tyrosine, histidine, asparagine, and aspartic acid. The abbreviations are: Q, 7-(3,4-*trans*-4,5-*cis*-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine; and G, guanine.

Table 2. Q-Hypomodification of tRNA in Chinese Hamster Cells Induced 7-Methylguanine

tRNA Source	Guanine Incorporation (pmoles/hr/A_{260} unit)
Chinese hamster cells	1.86
Plus 7-methylguanine	3.46
Minus 7-methylguanine	2.09
Escherichia coli	<0.2
Yeast	6.58

DISCUSSION

The potential value of modified nucleosides as biochemical markers for cancer can be seen in Fig. 1 and Fig. 2. Even at the time of cancer diagnosis, nucleoside excretion was elevated significantly for the NPC patient (Fig. 1), and this correlated with high serum antibodies to EBV antigens including antibody to the EBV DNase, a marker for NPC[22]. The AML patient exhibited even higher nucleoside excretion levels at the time of diagnosis (Fig. 2), although the pattern of elevated excretion was different. Unique excretion patterns may offer additional means to characterize specific cancers. The patient with AML also exhibited elevated peripheral blood cell adenosine deaminase activity, another potential biological marker for leukemia. The clinical value of monitoring various markers for leukemia and NPC is being assessed for both diagnostic and prognostic purposes.

The early increases in tRNA catabolism associated with neoplasia led to an examination of the potential role of the catabolites in neoplastic transformation. The discovery that chronic exposure to some, but not all, methylated purines derived from cellular RNA can transform normal diploid cells was quite perplexing[9,10]. However, it appears that the methylated purines may influence the expression of various transformed phenotypes. Removal of the transforming methylated purine at the appropriate time can result in reversal of expression of various transformed phenotypes, e.g., increased proliferative capacity (Fig. 3), anchorage independent growth, and tumorigenicity[21]. All transformed phenotypes are not reversed by removing the methylated

purine from a particular culture. However, it was demonstrated previously that the methylated purine-transformed cells exhibit elevated tRNA methyltransferase activity[9], and therefore, the endogenous methylated purine level may negate the need for an exogenous source.

The results obtained with the methylated purines suggested similarities to dedifferentiation associated with carcinogenesis. Since dedifferentiation reportedly involves changes in gene regulation at the post-transcriptional level[23], we have attempted to identify cellular targets for the methylated purines that might alter phenotypic expression by similar means. A proposed target was tRNA transglycosylase, since the enzyme from E. coli is inhibited by the methylated purine 7-methylguanine[18]. It was presumed that a major structural change (guanine vs Q-base) in transformed tRNA's for histidine, tyrosine, asparagine, and/or aspartic acid generated by inhibiting the transglycosylase might allow the altered tRNA isoaccepting species to translate disparate mRNA's more efficiently. As we have now found, 7-methylguanine does inhibit tRNA transglycosylase from a mammalian source, and it induces Q-hypomodification of cellular tRNA (Table 2) under conditions leading to the expression of transformation.

The question of whether Q-deficient tRNA's actually have some role in the expression of transformed phenotypes remains to be answered. However, it has been reported that reversing tRNA Q-deficiency in tumor cells by administration of purified Q-base was associated with diminution of tumor cell growth in vivo[24].

The numerous alterations in tRNA metabolism associated with neoplasia have led us to devise a scheme by which they may interrelate in the expression of carcinogenesis, and the proposed sequence of events is presented in Fig. 5. The induction (initiation) of carcinogenesis could be by any means. The subsequent events are then predicted to have cause and effect relationships, i.e., each change depicted would occur in order and be caused by the previous change. Therefore, soon after the induction event there would be an increase in tRNA methyltransferase activity which would result in an increase in methylated RNA catabolites. The higher endogenous levels of methylated purines would then modulate tRNA modification by inhibiting tRNA transglycosylase. The methylated purines might also modulate tRNA modification by acting as an alternate substrate for the transglycosylase or by other, as yet unidentified, means. Both the modulation and methylation steps would be involved in generating altered tRNA isoaccepting species, and these species might allow the translation of different mRNA's that are not translated efficiently by the normal tRNA population. It is then assumed that some of these translation products would be onco-developmental proteins. If

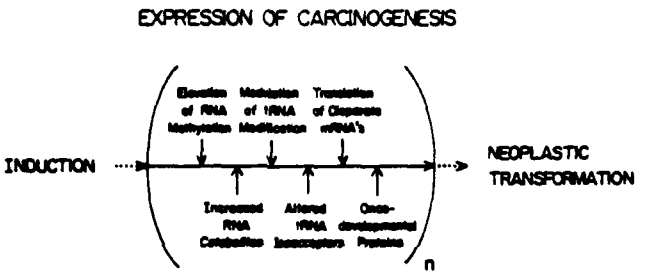

Fig. 5. Proposed model for the role of altered tRNA metabolism in the expression of carcinogenesis. Each event in the sequance is predicted to influence subsequent events, and the last may reinitiate the first.

any of these proteins were tRNA methyltransferases, and it is known that enzymes with different specificities appear[1], the cycle would repeat at a more aberrant level. By this means, the cycle could continue to generate accruing phenotypic alterations until neoplastic transformation is attained.

The proposed model offers an explanation for the general staging process of carcinogenesis. It also allows interpretation of the phenotypic reversibility phenomenon demonstrated for the exogenous methylated purines. A step back to the previous cycle might be possible by such a withdrawal, but the increased generation of endogenous methylated purines would block any further phenotypic reversion.

Certain of the individual points outlined in Fig. 5 have been proposed by other investigators to have a role in neoplastic transformation. However, linking the increased RNA catabolites to the expression of transformed phenotypes as well as the induction of tRNA hypomodification allowed us to formulate the comprehensive scheme presented. The hypothesis is being tested using a variety of model systems, and the similarities to promotion of neoplastic transformation are being studied. The numerous biochemical markers involved should greatly facilitate these investigations.

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EVALUATING TUMOR PROMOTER ACTIVITY IN VITRO WITH HUMAN DIPLOID FIBROBLASTS

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INTRODUCTION

Carcinogenesis is a long-term process involving the interaction of many endogenous and exogenous factors. The role of chemical pollutants in this process is complex and not well understood in most cases. The study of chemically-induced cancer can be subdivided into the stages of initiation and promotion. Classical initiation/promotion experimental protocols involve the application on mouse skin of sub-carcinogenic doses of an initiator [e.g. the polynuclear aromatic hydrocarbon (PAH) benzo(a)pyrene] followed by repeated applications of a non-carcinogenic tumor promoter [e.g. the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)]. In general, this initiation/promotion scheme *in vivo* can also be achieved *in vitro* with cultured rodent cells. The means by which phorbol esters and other agents promote carcinogenesis remains a subject of conjecture. Even in the absence of chemical carcinogen initiation *in vitro*, tumor promoters are able to induce reversible phenotypic changes in rodent cells that are characteristic of *in vitro* transformation; a phenomenon described as mimicry of transformation (1).

Although the rodent cell systems offer useful models for many studies of initiation/promotion and mimicry of transformation, the development of a human cell model would be highly desirable for the study of interactions of exogenous chemical agents in human cancer. Milo *et al.* (2) have demonstrated neoplastic transformation of normal human fibroblasts *in vitro* subsequent to treatment with complete chemical carcinogens, i.e., carcinogens that do not require promotion. However, classical initiation/promotion has not been achieved with human cells *in vitro* due to the lack of an appropriate model for the promotion stage. Diamond *et al.* (3) did report growth stimulation of normal human fibroblasts treated with TPA, but the transient 50% increase in the saturation density of TPA treated cells was attributed to a decrease in cell size induced by TPA. Few other studies on normal human cells in culture have described effects of tumor promoters that could be equated to mimicry of transformation. Tumor promoters alone do induce changes in the growth

characteristics of mutant human fibroblasts (4), and they also influence growth, differentiation, and DNA synthesis in human cancer cells (5,6,7).

Because few phorbol ester-induced effects on normal human cells have been demonstrated and those that have are not of the magnitude reported for rodent cells, it might be concluded that promotion of carcinogenesis is not relevant in man. Epidemiological studies suggest, however, that promotion is an important consideration. Therefore, we are developing a normal human cell culture system responsive to tumor promoters; one which should allow the study of mimicry of transformation as well as two-stage (initiation/promotion) carcinogenesis.

MATERIALS AND METHODS

Chemicals

The potential promoting compounds examined in this study included the phorbol esters TPA and 4-O-methyl-TPA (P.L. Biochemicals Inc., Milwaukee, Wisconsin). The former is the most potent tumor promoter on mouse skin, while the latter is an inactive structural analog (8). Mezerein, another plant diterpene and much weaker promoter than TPA on mouse skin (9), was obtained from Dr. L. David Tomei, Comprehensive Cancer Center, The Ohio State University. Other non-phorbol esters evaluated were anthralin, (1,8-dihydroxy-9-anthrone), 7,12-dimethylbenz(a)anthracene (7,12-DMBA) (both courtesy of Dr. George E. Milo, Department of Physiological Chemistry, The Ohio State University), and norharman, (9H-pyrido-[3,4-b]indole), (Sigma Chemical Co., St. Louis, Missouri). Anthralin is also a tumor promoter on mouse skin (10), while norharman, a component of tobacco smoke, is a co-mutagen (11). The PAH 7,12-DMBA is a complete carcinogen in rodent systems, but is inactive with normal human cells in vitro (12).

Cell Culture

Primary cultures of normal human fibroblasts were prepared from neonatal foreskin as described by Riegner et al. (13). The cells were grown in Eagle's minimal essential medium (GIBCO, Grand Island, New York) supplemented with 25 mM HEPES (pH 7.2), 0.2% sodium bicarbonate, 1.0 mM sodium pyruvate, gentomycin (5 µg/ml), 2X vitamins, 1X nonessential amino acids, and 2X aspartic acid, asparagine, histidine,

phenylalanine, and tyrosine. These modifications were found to enhance changes in phenotypic expression promoted by phorbol esters (manuscript in preparation), and the medium is referred to hereafter as promotion medium. The medium was also supplemented with either 20% fetal bovine serum (Reheis Chemical Co., Phoenix, Arizona, or Sterile Systems, Logan, Utah) or 20% bovine serum (Sterile Systems) for establishing primary cultures. At the first passage of the primary culture, the cells were split at a ratio of 1:4 into 25 cm^2 flasks, and the serum content of the medium was reduced to 10%. Once a week the cells were subcultured at a ratio of 1:10 and counted with a hemacytometer to determine saturation densities. Duplicate cultures were maintained for each treatment, and the cultures were fed with fresh promotion medium three days following subculture.

Each of the potential promoting agents was dissolved in acetone and added to promotion medium at a final concentration of 10^{-7}M and 0.01% acetone. For dose-response studies, promotion medium containing 10^{-6}M TPA and 0.1% acetone was prepared, from which serial dilutions were made. Control cultures were treated with the 0.01% or 0.1% acetone. Treatment of the cells with agents of interest was started at the first passage of the primary culture and continued throughout all subcultures.

DNA Synthesis

The system that we utilized to determine DNA synthesis is a modification of a procedure originated by Ball et al. (14) that allows us to grow, treat, and process human fibroblasts in glass scintillation vials. A primary culture of human fibroblasts was trypsinized and counted. Cultures were seeded by pipeting 2.0 ml aliquots of a suspension (0.25 X 10^5 cells/ml) into a series of sterile vials. When plated into the vials, promotion medium plus 20% BS was used. The cells were allowed to establish five days at which time they were confluent. The experiment was started by the addition of 10^{-7} M promoter and 0.01% acetone to the quiescent culture. Acetone alone (0.01%) was added to control cultures. To measure DNA synthesis, [^{3}H]thymidine (^{3}H-TdR) (Sp. act. 5 Ci/mmole) was added to each vial (0.5 μCi/vial) at various intervals. The cells were pulse labeled for 90 minutes and the medium was decanted.

Precursor incorporation was stopped by the immediate addition of 10 ml of ice-cold saline. The cells were gently rinsed 2 times. One ml of 1.5% (v/v) perchloric acid (PCA)

was applied to remove any unincorporated radioactive precursor and fix the cells to the surface of the vial. The acid was removed and counted to monitor any changes in the thymidine pool caused by exposure to promoter. Two further washes with 10 ml of 1.5% PCA were then applied. The vials were rinsed with 10 ml of 95% ethanol and inverted to dry. Finally, one ml of 5% PCA (v/v) was added to each vial and the samples were heated at 80°C for 40 minutes to hydrolyze nucleic acids. After cooling, scintillation fluid was added to each vial and the samples were counted.

RESULTS

Saturation Density

Significant changes in saturation density were observed when the cultured human fibroblasts were exposed continuously to TPA. The cells exhibited a loss of sensitivity to contact inhibition with an extreme degree of overgrowth. Figure 1 shows the saturation density dose response for cells treated with TPA. The saturation density for control cells was approximately 50,000 cells/cm^2 at all passages. Treatment with 10^{-8}M TPA gave the largest increase in saturation density above control values (up to 5-fold) in the experiment depicted. TPA at a concentration of 10^{-7}M was effective to a lesser degree, followed by 10^{-6} and 10^{-9}M TPA which produced nearly equal increases. A marked elevation in saturation density was observed for 10 independent primary cultures treated with 10^{-7}M TPA, with the maximum treatment values varying from 120,000 to 280,000 cells/cm^2. Substituting bovine serum for fetal bovine serum in the promotion medium had no significant effect on the TPA-induced response. Removal of TPA from the culture medium at any passage resulted in a return to near the control saturation density by the next passage.

At a concentration of 10^{-7}M, continuous treatment with 4-O-methyl-TPA, anthralin, norharman, or 7,12-DMBA had no effect on the saturation density of diploid human fibroblasts, i.e., the values were indistinguishable from the controls. However, when 10^{-7}M norharman was added to cells already being treated with 10^{-7}M TPA, a significant response was obtained (Figure 2). The norharman-induced increase was 2-fold greater than that induced by TPA alone, and it was transient in nature. It has not been possible to obtain similar effects when TPA and norharman were added concurrently at the first passage of the primary culture. In

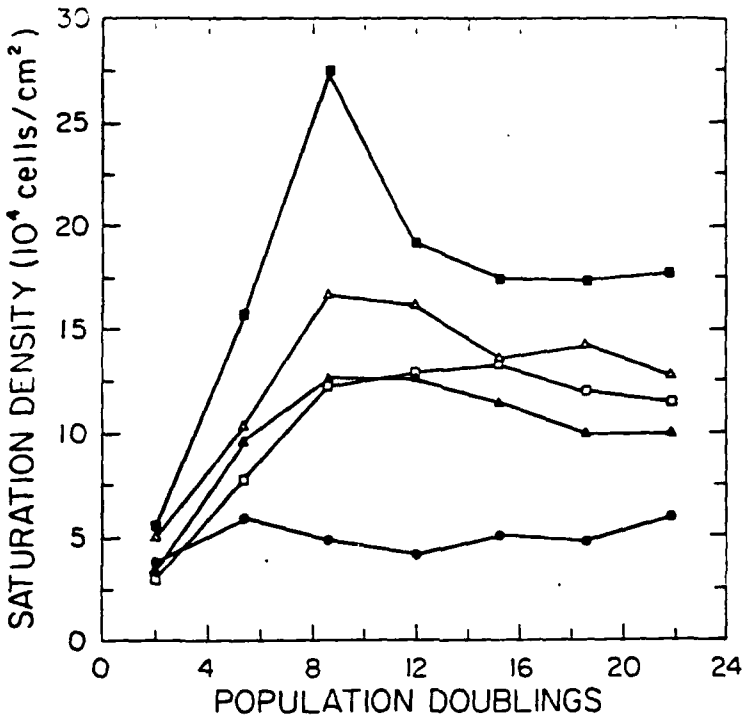

FIGURE 1. TPA dose-response curve. Human fibroblasts treated from passage one with TPA. The concentrations utilized were 10^{-6}M (▲), 10^{-7}M (△), 10^{-8}M (■), and 10^{-9}M (□). Control cells (●) were treated with 0.1% acetone.

a repeat of the experiment depicted in Figure 2, removal of norharman at any time after the synergistic increase in saturation density, resulted in a return to the TPA density by the subsequent passage.

Anthralin at a concentration of 10^{-7}M was also found to elevate the saturation density attainable in concert with 10^{-7}M TPA (Figure 3). As described earlier, 10^{-7}M anthralin alone was without effect. As shown in Figure 3, 10^{-7}M mezerein also promoted an increase in growth beyond

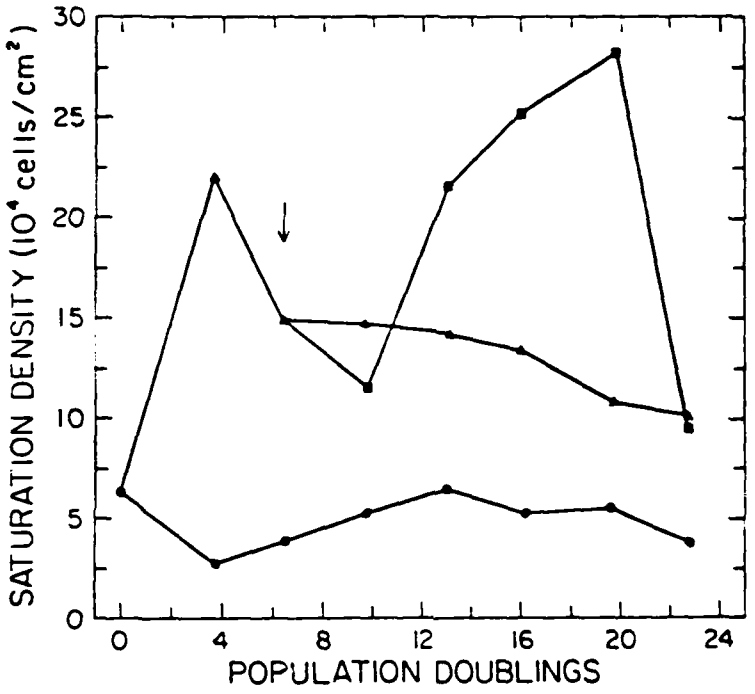

FIGURE 2. Effects of TPA plus norharman on saturation density. Cells were exposed continuously to 10^{-7}M TPA (▲) and after two passages to 10^{-7}M norharman (■). Control cells (●) were treated with 0.01% acetone. The arrow indicates the point at which norharman was added to duplicate cultures.

that obtained with 10^{-7}M TPA. However, continuous treatment with 10^{-7}M mezerein alone causes a reversible 2 to 3-fold increase in the saturation density of the normal human cells, so the effects of TPA plus mezerein may be additive.

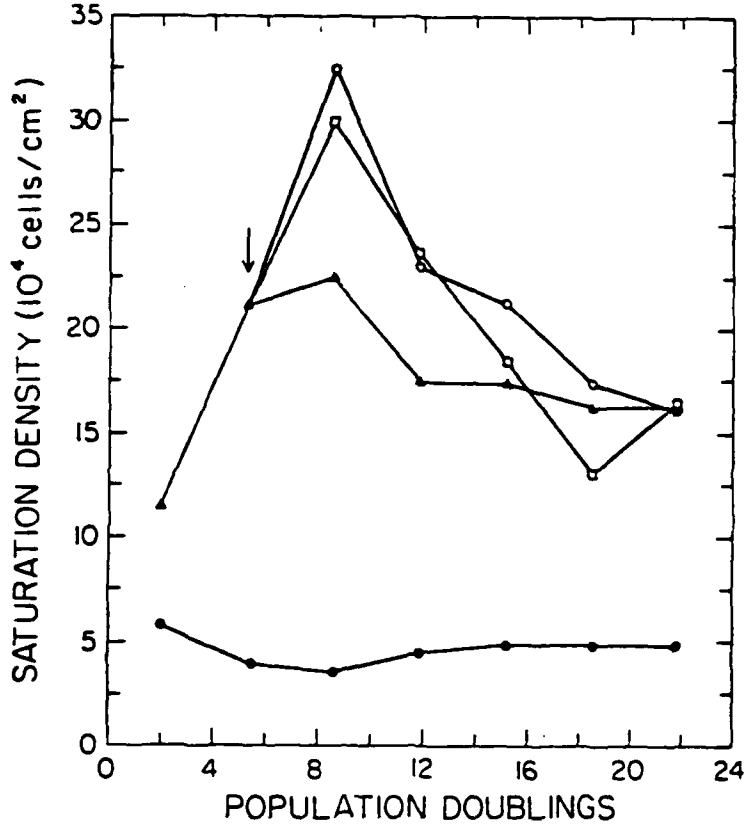

FIGURE 3. Effects of TPA plus anthralin or mezerein on saturation density. Addition of 10^{-7}M anthralin (□), or 10^{-7}M mezerein (○) to promotion medium of cells already exposed to 10^{-7}M TPA (▲) for three passages. Control cells (●) were treated with 0.01% acetone. The arrow indicates the point at which anthralin and mezerein were added to duplicate cultures.

DNA Synthesis

Changes in DNA synthesis were also observed with human skin fibroblasts treated with TPA (Figure 4). There was an initial inhibition of ^{3}H-TdR incorporation followed by a large stimulation of incorporation. TPA at a concentration of 10^{-7}M (the concentration depicted) gave the greatest and most prolonged inhibition of DNA synthesis, followed by 10^{-8} and 10^{-9}M. The DNA stimulatory response was nearly equal for 10^{-7} and 10^{-8}M TPA and was undetected for 10^{-9}M TPA. However, the TPA-induced stimulatory (mitogenic) response was less reproducible and more influenced by culture conditions than the inhibitory response. Treatment of human diploid fibroblasts with 10^{-7}M 4-O-methyl-TPA, norharman, anthralin, or 7,12-DMBA had no effect on DNA synthesis. Treatment with norharman or anthralin in conjunction with TPA also had no effect on the inhibition or stimulation of DNA synthesis caused by TPA. Mezerein at a concentration of 10^{-7}M inhibited DNA synthesis in a transient manner, similar to TPA.

DISCUSSION

It was established in this investigation that under appropriate culture conditions continuous exposure to TPA or mezerein significantly alters phenotypic and biochemical properties of low passage human diploid fibroblasts. An acute TPA-induced mitogenic response similar to that depicted in Figure 4 has been demonstrated previously with normal human cells (15), but reported phenotypic changes for these cells are minimal (3). The 50% increase in saturation density induced by TPA in the previous studies was attributed to a somewhat smaller cell size (3). Even then, a 50% increase above the normal cell density for human cells of 40,000 to 50,000 cells/cm^2 would yield only 60,000 to 75,000 cells/cm^2. That is a hardly comparable to levels up to 280,000 cells/cm^2 demonstrated under the conditions prescribed in this investigation. Although a significant elevation in saturation density was induced by TPA, some variability was observed in the magnitude and timing of the response. In addition, the secondary response to norharman, anthralin, and mezerein in TPA treated cultures was greatest when the cells were highly responsive to TPA. This variability suggests differences in promoter sensitivity among primary human skin cell cultures; an aspect currently being explored.

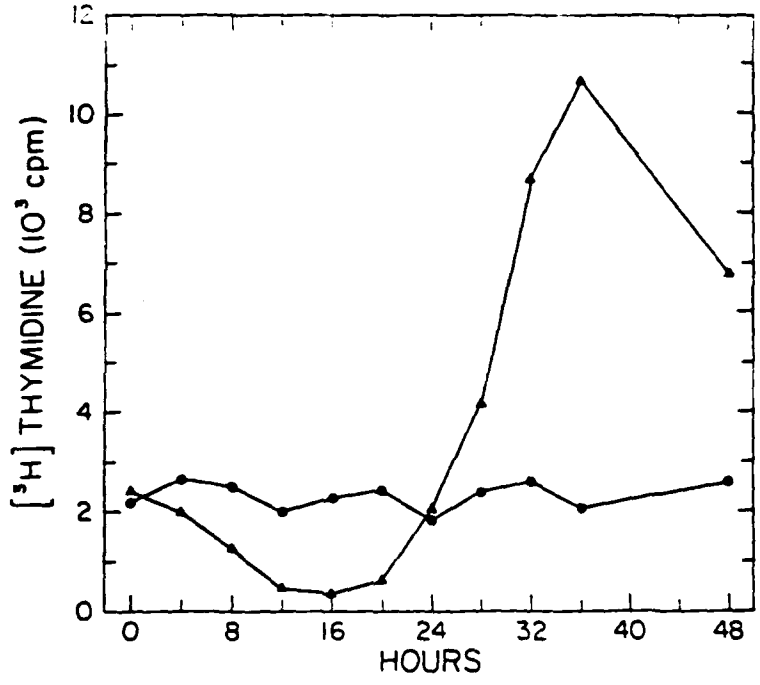

FIGURE 4. Effects of TPA on DNA synthesis. Primary cultures of normal human fibroblasts were seeded and grown in glass scintillation vials (Materials and Methods). Five days later the confluent, quiescent cultures were treated with 10^{-7}M TPA (▲) or 0.01% acetone (●) at time zero. At 4 hour intervals the cells were pulse labeled with ^{3}H-TdR.

The only two agents that were able to induce mimicry of transformation by themselves, TPA and mezerein, were also the only two that inhibited DNA synthesis immediately after exposure. Peterson et al. (16) suggested that the transient inhibition of DNA synthesis in cultured mouse fibroblasts correlated with promoter activity of various phorbol esters; an observation in agreement with our human fibroblast results. Specificity is indicated by the fact that 4-O-methyl-TPA, anthralin, norharman, or 7,12-DMBA alone neither inhibited DNA synthesis nor promoted a loss of

contact inhibition of growth. Therefore, evaluating agents for their ability to inhibit DNA synthesis in human diploid fibroblasts following acute exposures may offer a rapid means to identify "complete promoters" for these cells.

The effects of norharman and anthralin in conjunction with TPA are more complex. Apparently these compounds are "incomplete promoters" or "co-promoters" for inducing mimicry of transformation of normal human cells. The promotion of mouse skin tumors was reported by Slaga et al. (17) and Furstenberger et al. (18) to be at least a two-stage process, with some agents functioning as second stage promoters following TPA exposure. Our in vitro human cell results are consistent with anthralin and norharman being second stage promoters, but other possibilities have not been totally ruled out. Mezerein may function both as a weak complete promoter and a second stage promoter.

The biochemical and phenotypic changes induced in human diploid fibroblasts by putative tumor promoters may aid in establishing agents which are relevant to human carcinogenesis. In addition to further investigations of mimicry of transformation, conditions are being defined to examine two-stage carcinogenesis with the human cells in vitro. By this means we hope to better understand the role of exogenous chemical agents in cancer.

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In Vitro (in press)

ALTERED GROWTH PROPERTIES OF NORMAL HUMAN CELLS INDUCED BY PHORBOL 12,13-DIDECANOATE

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Running head: Effects of PDD on human cells.

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SUMMARY

The tumor promoter phorbol 12,13-didecanoate (PDD) significantly alters the growth properties of early passage normal human skin cells *in vitro* in culture medium supplemented with elevated concentrations of selected amino acids. Continuous treatment of cells with 10^{-7} or 10^{-8}M PDD results in a 5 to 10-fold increase in saturation density at early passages followed by a long-term 2 to 4-fold increase. The PDD-treated cultures remain in exponential growth at cell densities greater than 10-fold higher than the control cultures. Removal of PDD from the culture medium while the cells are at a high cell density results in a return to near-normal saturation density by the subsequent passage. Anchorage-independent growth of normal human cells in methylcellulose is also promoted by PDD in a dose-dependent manner, with prior subculturing in the presence of PDD being required for maximal colony formation. The structural analog 4α-phorbol 12,13-didecanoate (4α-PDD) fails to elicit similar cellular responses.

Key words: Tumor promoters; human skin cells; mimicry of transformation.

INTRODUCTION

Investigations of staging of carcinogenesis, based on the two-stage mouse skin model (1), have been reported with various cell culture systems (2-7). Tumor promoters elicit numerous changes in cell growth characteristics in these systems even in the absence of initiation of carcinogenesis; an *in vitro* phenomenon referred to as mimicry of transformation (8). However, there have been few reports of phorbol ester tumor promoters significantly altering the growth properties of normal human cells *in vitro*, thereby greatly impeding the study of promotion of carcinogenesis as it relates specifically to man. A slight decrease in the size of diploid human fibroblasts induced by phorbol esters was reported to allow a marginal increase (up to 50%) in the saturation density attainable under standard cell culture conditions (9). At low cell densities, phorbol esters reportedly inhibit the proliferation of normal human cells which is in contrast to the expected *in vitro* response for a tumor promoter (10). The tumor promoters do induce various changes in the *in vitro* growth characteristics of mutant human fibroblasts and human cancer cells that are more characteristic of promotion as defined for cultured rodent cells (10-14).

The expression of transformed phenotypes by human skin fibroblasts subsequent to treatment with chemical or physical carcinogens was reported by Milo *et al.* (15) to be augmented by supplementing the culture medium with excess (8X) nonessential amino acids. While examining the long-term effects of the tumor promoter PDD on normal human cells in culture, it was observed that significant changes in phenotypic expression (e.g. increased saturation density) were obtained in the same medium. Partial characterization of PDD-induced changes in the growth properties of diploid human cells *in vitro* is the subject of this report.

MATERIALS AND METHODS

Cell culture. Primary cultures of normal human cells were prepared from neonatal foreskin as described by Riegner et al. (16), with each primary indicating an individual donor. At the first passage of the primary culture, the fetal bovine serum (Reheis, Kankakee, IL, or Sterile Systems, Logan, UT) content in Eagle's minimal essential medium (MEM) (GIBCO, Grand Island, NY) was reduced from 20% to 10%, and it was maintained at that level for subsequent passages. The MEM was also supplemented with 25 mM HEPES buffer (pH 7.2), 2 mM glutamine, 1 mM sodium pyruvate, gentamicin (50 μg/ml), 0.2% sodium bicarbonate, 2X vitamins, and either 8X nonessential amino acids or 1X nonessential amino acids plus 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine. In some experiments (not described), fibroblasts and epithelial cells were separated by selective trypsinization as described by Milo et al. (17), so each cell population could be treated individually with the phorbol ester. However, this step was not included routinely, since it was important to add the phorbol ester to the cells as early as possible. In addition, only the fibroblasts, which predominate in the primary culture, gave phorbol ester-induced responses characteristic of mimicry of transformation while epithelial cell growth (stratification) was inhibited. Addition of phorbol esters to matched cultures was initiated at the first passage of the

ry culture for all experiments described. Post-confluent cell populations in duplicate were trypsinized, and cells were counted with a hemacytometer to establish saturation densities. Cell volume distribution data for phorbol ester-treated and untreated cells was obtained and processed with a Coulter Counter Model ZBI equipped with a C-1000 Channelyzer and Accucomp computer interface.

For the determination of anchorage-independent growth, cell culture dishes (60 mm) were overlaid with 5 ml of 1.0% Difco agar in MEM supplemented with 20% fetal bovine serum, 20% tryptose phosphate broth, 1 mM sodium pyruvate, 1X nonessential amino acids, 2X vitamins, and 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine. The cells (10^5) in single cell suspensions were overlaid in duplicate in 10 ml of 1.3% methylcellulose in S-MEM (MEM for suspension culture) supplemented as above, but without tryptose phosphate broth. Colonies were scored after 21 days.

Phorbol esters. PDD and 4α-PDD (P-L Biochemicals, Milwaukee, WI) were dissolved in acetone. The acetone concentration was 0.01% in phorbol treated and untreated cultures.

RESULTS

Increased saturation density. The saturation density of normal human cells cultured in the medium containing 8X nonessential amino acids was significantly increased by 10^{-7}M PDD (Figure 1A). The saturation density for the normal control cells was approximately 4 x 10^4 cells cm^{-2} at most passages, while the PDD-treated cells had saturation densities 2 to 4-fold higher than controls at each passage over most of the lifetime of the cultures. The specificity of the effect for a tumor promoting phorbol ester is demonstrated by the fact that 4α-PDD gave results similar to the untreated controls. PDD has induced comparable elevations in saturation density at successive passages in four different primary cultures in medium supplemented with 8X nonessential amino acids. This 2 to 4-fold increase in saturation density was obtained in spite of the fact that 10^{-7}M PDD reduced the cloning efficiency 20 to 30% with all primary cultures under the conditions employed. Lower PDD concentrations (10^{-8} to 10^{-10}M) had no effect on the cloning efficiency of human skin cells (data not presented).

While examining the effects on saturation density of supplementation with various other combinations and concentrations of amino acids, an even greater initial increase was observed for normal cells treated with 10^{-7}M PDD in medium containing 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine (Figure 1B). Cell densities in the PDD-treated cultures reached levels 7-times those of the acetone and 4α-PDD controls in the experiment depicted, and removal of PDD from the culture medium resulted in a return to near-normal saturation density within a few population doublings (Figure 1B). Similar results have been obtained with ten different primary cultures treated with 10^{-7} or 10^{-8}M PDD, with the early increases in saturation density ranging from 5 to 10-fold. Eliminating the feeding step after 3 days as described in

Figure 1B or substituting bovine serum for fetal bovine serum in the culture medium for the duration of the experiment did not alter the results significantly. Although the timing of the transient increase in density has been somewhat variable from one primary culture to another, all the cultures have exhibited a long-term 2 to 4-fold elevation subsequently, similar to that seen in 8X nonessential amino acids. Two of the ten primary cultures evaluated in the 2X supplemented medium also had PDD added after the control cells had undergone 15 to 20 population doublings in culture, and under these circumstances, only 2-fold increases in density were obtained. Both of these primaries had yielded saturation densities greater than 5-fold above the untreated control cells when the phorbol ester was added at the first passage.

The increased saturation density induced by PDD can be seen in Figure 2. The PDD-treated cells (Figure 2B) exhibited an extreme degree of overgrowth, while the control (Figure 2A) and 4α-PDD-treated cells (Figure 2C) did not, i.e., the latter were responsive to density-dependent growth inhibition. The overgrowth of the cells treated with PDD was still readily apparent even though the saturation density attained had decreased from 7-times the control on the previous passage to only 3-times when the photomicrographs were taken (Figure 1B). The cells in treated and untreated cultures retained predominantly a fibroblast-like morphology, but the PDD-treated cells did appear smaller by microscopic observation at both low and high cell densities. Cell sizing measurements for PDD-treated and untreated human cells gave results for PDD-induced decreases in cell volume ranging from negligible to 40% depending on the phorbol ester concentration, duration of treatment, and cell growth phase. Under the conditions depicted in Figure 2B, cell size was decreased 30-40% by PDD.

The degree to which PDD caused a loss of sensitivity to contact inhibition of cell division of normal human cells is demonstrated in Figure 3. Cells treated with 10^{-8}M PDD remained in exponential growth at significantly (>10-fold) higher cell densities than untreated cells. The slight decrease in the PDD-treated cell density after day 6 in the experiment depicted (Figure 3) was associated with the appearance of floating, non-viable cells. No further increase in dead cells or decrease in density was then observed after 7 to 8 days, i.e., the cells had entered the stationery, non-proliferating phase. The rapid cessation of growth by the PDD-treated cells depicted in this experiment (Figure 3) together with the obvious lack of density-dependent inhibition of growth (Figure 2B) suggests that some essential nutrient may become limiting in the culture environment.

Anchorage-independent growth. If the normal human skin cells were exposed to PDD continuously under the prescribed conditions, anchorage-independent growth was also promoted in a dose-dependent fashion (Figure 4). As shown, few colonies were obtained when the cells were passaged only 2 times (ca. 7 population doublings) with PDD prior to seeding in methylcellulose, while the number increased significantly after 4 passages (ca. 14 population doublings). However, no further increase in the number of colonies was obtained after 6 passages (ca. 20 population doublings). In the latter case, the control cells and cells exposed to 10^{-8}M PDD were also seeded in methylcellulose containing 10^{-7}M PDD to verify the importance of prior subculturing in PDD to obtain significant anchorage-independent growth. The average colonies per plate for the control cells increased only from 5.5 to 21 under these conditions, while the average for the PDD-treated cells increased from 7.5 to 83.5. In all cases, the few cell colonies obtained without added promoter were much smaller than those with promoter.

DISCUSSION

The induction of transformed phenotypes in cultured normal human cells by phorbol ester tumor promoters has not been described although such promoter-induced mimicry of transformation is well known in cultured rodent cells (8). The data presented in this report demonstrate that under appropriate culture conditions, tumor promoters can also induce the expression of transformed phenotypes in early passage human skin cells. The loss of density-dependent growth inhibition in monolayer culture as well as the anchorage-independent growth induced by the phorbol ester PDD are characteristic transformed phenotypes expressed by human skin cells during neoplastic transformation induced by chemical and physical carcinogens *in vitro* (15). Therefore, PDD elicits a mimicry response in the absence of carcinogen initiation.

The increase in cell density induced by chronic exposure of human cells to PDD was appreciable (Figure 1). Although it was suggested that smaller cell size ($\leq$30%) might explain the 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced increases in saturation density ($\leq$50%) reported by Diamond *et al.* (9), it obviously could not explain the PDD-induced increases of up to 1,000% in the present investigation. While similar decreases in cell size were obtained under some conditions with PDD, the 2 to 10-fold increase in saturation density induced by PDD was not due to significantly more cells per unit area on the surface of the flask. As with carcinogen transformed human cells (15), the PDD-treated cells were no longer sensitive to density-dependent growth inhibition (Figure 2B). This is in contrast to the reported phorbol ester-induced inhibition of cell proliferation for sparse cultures of normal human cells under standard culture conditions (10). However, as with most studies involving cultured human cells, the phorbol ester utilized previously

was TPA and the treatment period was short-term. TPA has been reported both to inhibit and to stimulate proliferation of mouse fibroblasts depending on the culture conditions and timing employed (18). PDD was also somewhat inhibitory during the first exposure to the human cells in this study, and although quantitative comparisons were not undertaken, PDD always gave consistent increases in saturation density while TPA did not (data not presented).

Anchorage-independent growth is not a common tumor promoter-induced phenomenon for normal rodent cells or normal human cells under standard culture conditions (3,4,12,19), i.e., it is not a common characteristic of mimicry of transformation for normal cells (8). However, some carcinogen-initiated mouse epidermal cell lines and adenovirus-transformed rat embryo fibroblasts do exhibit increased anchorage-independent growth in the presence of phorbol ester and non-phorbol ester tumor promoters, and the response occurs in a dose-dependent manner (3,4,20). Normal human skin cells are also subject to phorbol ester-induced anchorage-independent growth under the prescribed culture conditions (Figure 4). This response is also dose-dependent, but whereas the rodent cells only require short-term exposure to the phorbol ester, the human cells require long-term exposure (Figure 4). Considering that repeated applications of a phorbol ester are necessary for tumor promotion on mouse skin following carcinogen initiation (1), the induction of anchorage-independent growth by chronic exposure of normal human cells to PDD in vitro may be analogous to mimicry of tumor formation in vivo.

Further changes in the cell culture environment may make it possible to obtain even greater PDD-induced alterations in the growth properties of human diploid cells. However, variations in amino acid concentrations in the culture medium appear to be important to obtain reproducible effects from one primary

culture to another. In addition, starting the phorbol ester treatment at the first passage of the primary culture enhances the observable cellular responses significantly. The *in vitro* transformation protocol of Milo *et al.* (15) utilizing the same cell type, requires that the cells undergo only a few population doublings before carcinogen treatment in order to attain neoplastic transformation. Therefore, human cell populations may become refractory to initiation and promotion of carcinogenesis with prolonged culture, i.e., they are modulated by the tissue culture environment. The different treatment protocol utilized might thus explain the significantly greater phenotypic response to a phorbol ester tumor promoter than demonstrated previously for normal human cells. Using this new protocol, it may be possible to study promotion of carcinogenesis following chemical carcinogen initiation using normal human cells *in vitro*.

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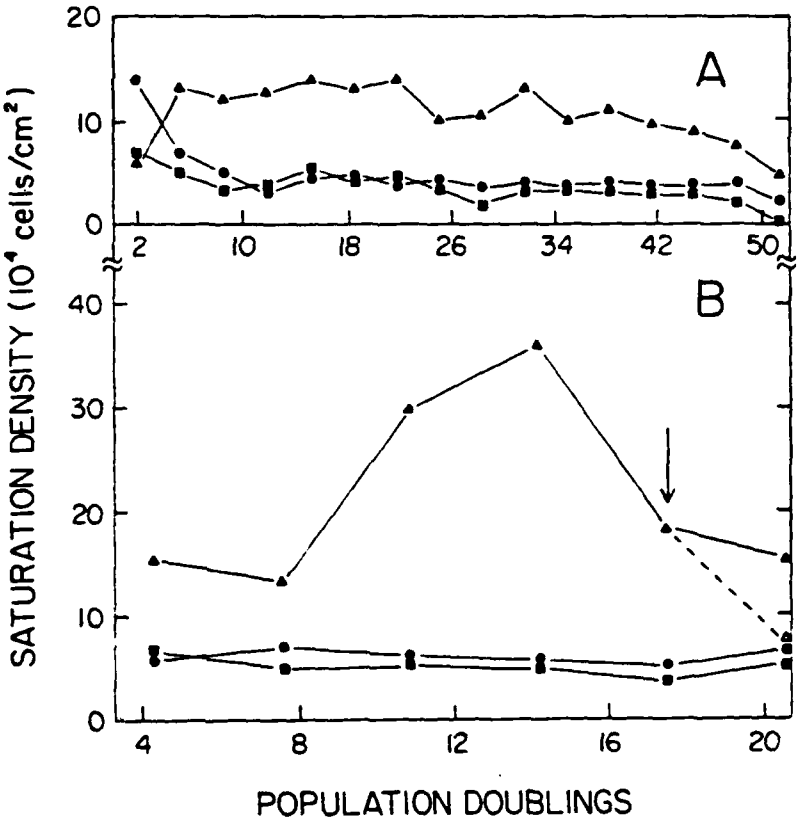
Figure 1. Saturation densities of human diploid cells exposed continuously to phorbol esters. The cell populations are: Control (●), 10^{-7}M PDD (▲), and 10^{-7}M 4α-PDD (■). (A) Human skin cells cultured in medium supplemented with 8X nonessential amino acids. The primary culture was split at a ratio of 1:4 after which the treated and untreated populations were subcultured at a ratio of 1:10 at 7 day intervals. The ordinate indicates control population doublings 2-51.5. (B) Human skin cells cultured in medium supplemented with 1X nonessential amino acids plus 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine. Cell populations were subcultured at a ratio of 1:10, were fed with fresh medium after 3 days, and were enumerated after 7-8 days. The ordinate indicates control population doublings 4.3-20.8. The arrow denotes the subculture at which PDD was removed from duplicate cell populations (Δ;dashed line), and also when additional post-confluent cultures were stained and photographed (Figure 2).

Figure 2. Photomicrographs of control and phorbol ester-treated human cells. Post-confluent cultures (Figure 1B, control population doubling level 17.5) were fixed in methanol 9 days after subculture and were stained with May-Grünwald Giemsa. (A) Control, (B) PDD-treated, and (C) 4α-PDD-treated. (X200).

Figure 3. Growth curves for human diploid cells grown in the absence and presence of PDD. The cell populations are: Control (●) and 10^{-8}M PDD (▲). Secondary post-confluent cultures (cultures having been exposed to 0.01% acetone ± 10^{-8}M PDD for one passage) in medium supplemented with 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine were subcultured 1:10 (Control) and 1:30 (PDD) on day 0. Duplicate cultures were trypsinized and counted with a hemacytometer each day.

Figure 4. Dose dependence of PDD-induced anchorage-independent growth of normal human cells. The cells were seeded in methylcellulose after two passages (O), four passages (●), and six passages (▲) in 0, 10^{-9}, 10^{-8}, or 10^{-7}M PDD. Conditions as described in Figure 1B for growth and subculturing of cells and as in MATERIALS AND METHODS for assessing anchorage-independent growth. The methylcellulose overlay medium was supplemented with the indicated concentrations of PDD.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B
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34
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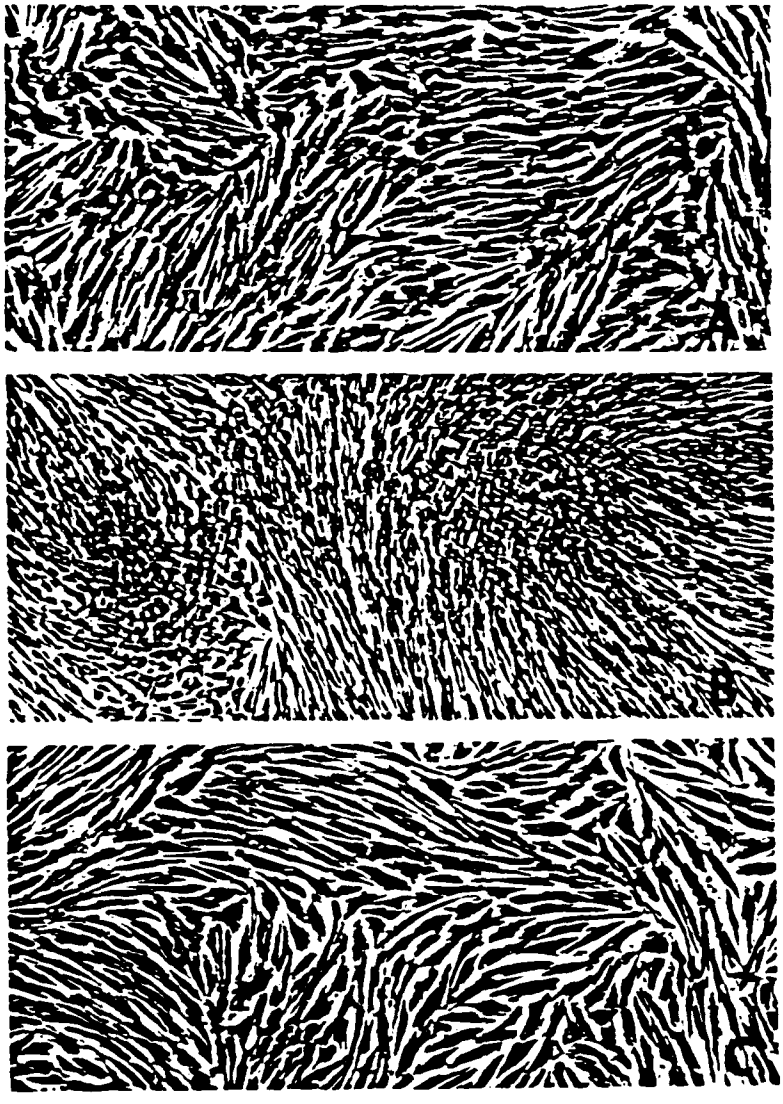
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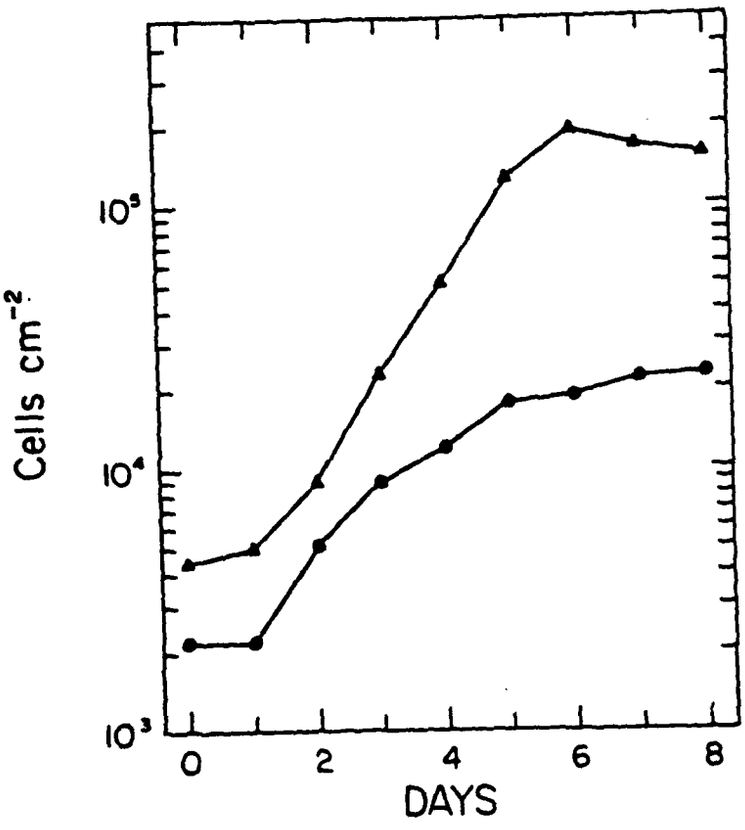
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10^4
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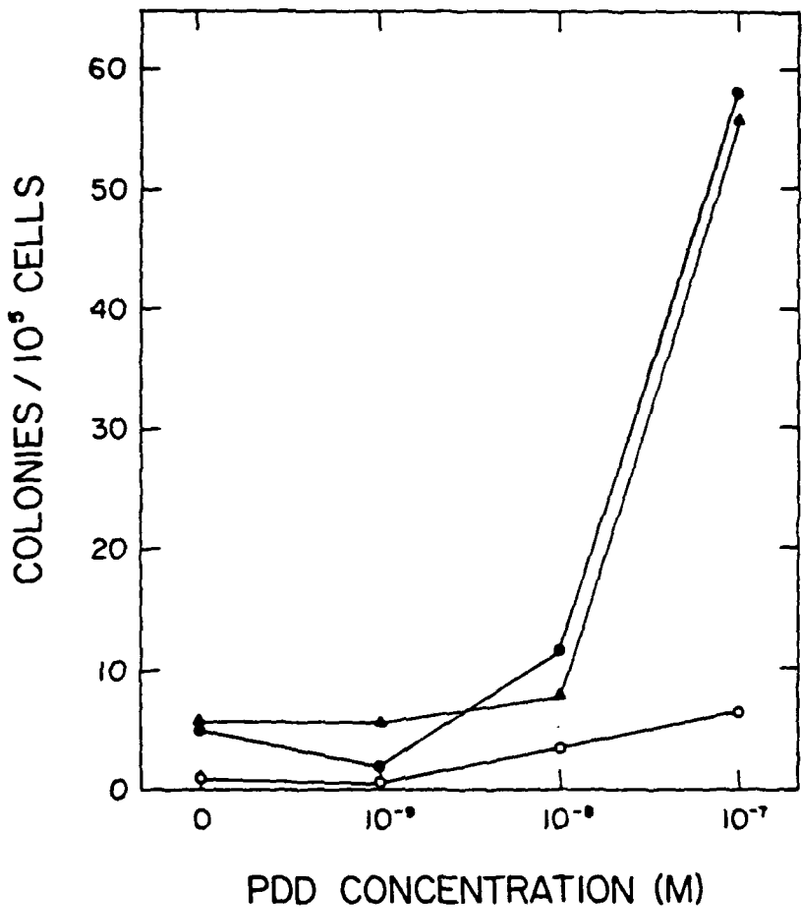
DAYS


COLONIES / 10^5 CELLS
60
50
40
30
20
10
0
0
10^{-9}
10^{-8}
10^{-7}
PDD CONCENTRATION (M)

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INOSINE BIOSYNTHESIS IN TRANSFER RNA BY AN ENZYMATIC INSERTION OF HYPOXANTHINE*

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Running head: Enzymatic insertion of hypoxanthine into tRNA

SUMMARY

An enzyme was discovered which incorporates hypoxanthine into mature tRNA macromolecules. This enzyme is postulated to be similar to tRNA-guanine ribosyltransferase which inserts queuine[1] into the first position of the anticodon of four tRNAs. The hypoxanthine incorporating enzyme has been assayed in extracts of rat liver and cultured human leukemia cells, and it has been resolved from tRNA-guanine ribosyltransferase by DEAE-cellulose column chromatography. The enzyme assay is based on the incorporation of radiolabelled hypoxanthine into unfractionated heterologous tRNA, and the reaction rate is proportional to the amount of added enzyme extract. Hydrolysis of the radiolabelled tRNA and analysis of the nucleoside composition yields inosine (the nucleoside of hypoxanthine) as the only radiolabelled product. It is proposed that the enzyme, a tRNA-hypoxanthine ribosyltransferase, is responsible for the biosynthesis of inosine in the anticodon wobble position of specific tRNAs, resulting in greatly expanded codon recognition by these tRNAs.

Extensive post-transcriptional modification of tRNA generates macromolecules containing an array of altered purines and pyrimidines. One modified nucleoside found exclusively in the anticodon of tRNAs is inosine. Inosine has been shown to occur in the first position of the anticodon of tRNA isoacceptors accommodating eight different amino acids, i.e., some of the tRNA isoacceptors for alanine, arginine, isoleucine, leucine, proline, serine, threonine, and valine contain inosine (1). The wobble hypothesis of codon-anticodon pairing proposed by Crick (2) states that inosine in the first position of the anticodon of tRNAs could base pair with uridine, cytidine, or adenosine in the third position of appropriate codons in mRNA, while standard base pair interactions would be maintained in the other two positions. Adenosine in the tRNA wobble position would base pair only with uridine, guanosine with cytidine or uridine, cytidine with guanosine, and uridine with adenosine or guanosine (2). Therefore, inosine in the anticodon expands the codon recognition potential of a particular tRNA.

The precise details of inosine biosynthesis in tRNA have not been established. Kammen and Spengler (3) demonstrated that inosine must be generated as a post-transcriptional modification to the tRNA macromolecule, but an enzyme capable of carrying out this modification reaction has not been described. Kammen and Spengler (3) could find no evidence for a postulated tRNA anticodon-specific adenosine deaminase generating inosine in the macromolecule, so it was necessary to investigate alternate biosynthetic mechanisms.

The only macromolecular purine modification which has been shown to occur by direct base replacement is the one involved in generating the nucleoside queuosine in the anticodon wobble position of tRNAs for aspartic acid, asparagine, histidine and tyrosine (4-7). The enzyme tRNA-guanine

ribosyltransferase (EC 2.4.2.29) catalyzes the exchange of guanine in the primary transcript for the highly modified base queuine, thereby giving rise to the nucleoside queuosine. Evidence is presented here that inosine biosynthesis in tRNA occurs by a similar mechanism, whereby hypoxanthine is inserted directly into tRNA macromolecules.

EXPERIMENTAL PROCEDURES

Enzyme Assays--The assay conditions for tRNA-hypoxanthine ribosyltransferase were a modification of those of Howes and Farkas (5) for tRNA-guanine ribosyltransferase. The standard reaction mixture contained 10 µmol Tris-HCl (pH 7.4), 53 µmol KCl, 2 µmol $MgCl_2$, 0.2 µmol EDTA, 5 µmol 2-mercaptoethanol, 6 µmol allopurinol, 1.0 A_{260} unit of Escherichia coli tRNA, 1 µCi [8-^{3}H]hypoxanthine (1 Ci/mmol) and enzyme extract in a total volume of 0.6 ml. The reaction mixtures in triplicate were incubated at 37°C for 30 to 60 minutes. Radiolabelled tRNA was precipitated with ice-cold 5% trichloroacetic acid and collected on glass fiber filters for scintillation counting (8). Similar assay conditions were employed for tRNA-guanine ribosyltransferase, but with yeast tRNA and [8-^{3}H]guanine as substrates and without the xanthine oxidase inhibitor allopurinol (5,8).

DEAE-Cellulose Column Chromatography--Conditions utilized were similar to those of Shindo-Okada et al. (9) for rat liver tRNA-guanine ribosyltransferase. Livers excised from two Sprague-Dawley rats were immediately homogenized at 4°C in 4-5 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol and 10% glycerol. The homogenate was centrifuged at 30,000 x g for 20 minutes at 4°C, and the resulting supernatant was then centrifuged at 105,000 x g for 60 minutes at 4°C. The final supernatant was

loaded onto a DEAE-cellulose column (25 x 2.4 cm) equilibrated with the Tris-HCl buffer described above. The column was washed with 200 ml of buffer, and then was eluted with a 200 ml linear gradient of 0-0.6 M NaCl in Tris-HCl buffer. Fractions (5 ml) were collected and aliquots of 0.1 ml were assayed for tRNA ribosyltransferase activities.

Cultured Cells--Human promyelocytic leukemia (HL-60) cells, obtained from Dr. Robert Gallo at the National Cancer Institute, were grown in suspension culture in RPMI-1640 medium supplemented with 15% fetal bovine serum. Human leukemic T-lymphoblasts (CCRF-CEM) in suspension culture were grown in Eagle's MEM supplemented with 10% bovine serum. Cells harvested by centrifugation were homogenized in 2-4 volumes of Tris-HCl buffer (described above). The homogenate was centrifuged at 30,000 x g for 30 minutes at 4°C, and the supernatant was utilized in the enzyme assays.

HPLC of tRNA Nucleosides--The enzyme assay was performed as described above, but with 5 A_{260} units of E. coli tRNA, 2 µCi [8-H^3]hypoxanthine, and 0.1 mg protein from an HL-60 cell extract. After a 60 minute reaction time, the tRNA-hypoxanthine ribosyltransferase reaction was terminated by the addition of three volumes of ice-cold 50 mM sodium acetate buffer (pH 4.5) containing 5 mM 2-mercaptoethanol, 5 mM EDTA, 10 mM $MgCl_2$, and 0.3 M NaCl. The radiolabelled tRNA was isolated by DEAE-cellulose column chromatography (10), and was recovered by ethanol precipitation. The precipitate was dried in vacuo and redissolved in 50 µl of water. The tRNA was then subjected to enzymatic hydrolysis with Penicillium citrinum nuclease P1 and E. coli alkaline phosphatase as described by Gehrke et al. (11), and was analyzed for radiolabelled nucleoside content by reversed-phase HPLC (12). The column used was an IBM-ODS (4.5 x 250 mm) with an initial mobile phase of 94.5% 0.1 M

$NH_4H_2PO_4$ (pH 5.1) and 5.5% methanol. After 18 minutes, the methanol concentration was increased to 8.5%. Fractions were collected and counted by liquid scintillation.

Materials--*E. coli* tRNA was obtained from Biogenics Research Corp., Chardon, Ohio, while yeast tRNA and *P. citrinum* nuclease P1 were obtained from Boehringer Mannheim, Indianapolis, Indiana. *E. coli* alkaline phosphatase was obtained from Sigma Chemical Co., St. Louis, Missouri. The radiolabelled purines [8-^{3}H]hypoxanthine and [8-^{3}H]guanine were both purchased from Amersham, Arlington Heights, Illinois. Fetal bovine and bovine serum were obtained from Sterile Systems, Logan, Utah, while the cell culture media RPMI-1640 and Eagle's MEM were purchased from GIBCO, Grand Island, New York. The IBM-ODS HPLC column was purchased from IBM Instruments, Inc., Wallingford, Connecticut.

RESULTS

An enzymatic activity was detected in rat liver and cultured human leukemia cells (CCRF-CEM and HL-60) which incorporates hypoxanthine into unfractionated tRNA. The rat liver enzyme was resolved from tRNA-guanine ribosyltransferase by DEAE-cellulose column chromatography (Fig. 1). The tRNA-guanine ribosyltransferase eluted at a NaCl concentration of approximately 0.04-0.06 M as previously reported by Shindo-Okada *et al.* (9), while the putative tRNA-hypoxanthine ribosyltransferase eluted at a concentration of 0.25-0.30 M NaCl (Fig. 1). The latter enzyme is very labile, with a half-life *in vitro* of only a few hours, making further purification difficult. Limited cross reactivity between substrates was exhibited by the enzymes, with the early eluting tRNA-guanine ribosyltransferase demonstrating substrate preferences *in vitro* for

yeast tRNA and guanine as previously reported (9), while the later eluting enzyme preferred E. coli tRNA and hypoxanthine (Fig. 1).

The product of hypoxanthine insertion into E. coli tRNA was established using the enzyme from human promyelocytic leukemia (HL-60) cells. After radiolabeling with [8-^{3}H]hypoxanthine, the tRNA was isolated and subjected to enzymatic hydrolysis. The nucleoside composition was then evaluated by HPLC (Fig. 2). Inosine was the only radiolabelled product obtained, thereby demonstrating a covalent modification to the macromolecule. Similar results were obtained with the enzyme from rat liver, but the liver enzyme studies were more difficult due to the high level of enzymes capable of metabolizing the substrates.

The rate of the hypoxanthine insertion reaction was proportional to the amount of added enzyme extract as shown with the enzyme from human leukemic T-lymphoblasts (CCRF-CEM) (Fig. 3). In addition, the time course of the reaction was linear for 30 to 40 minutes with the CCRF-CEM and HL-60 enzymes, and for 60 to 90 minutes with the rat liver enzyme (data not shown).

The hypoxanthine accepting ability of various unfractionated tRNA substrates for the CCRF-CEM enzyme are indicated in Fig. 4. E. coli tRNA was a better substrate than yeast tRNA for the human leukemia cell enzyme (Fig. 4) as it was for the rat liver enzyme (Fig. 1). The need for a heterologous tRNA substrate is also demonstrated in Fig. 4, since the leukemia cell enzyme was unable to insert appreciable hypoxanthine into the homologous leukemia cell tRNA.

Kinetic evaluations of the CCRF-CEM enzyme in a crude cell extract yielded a K_m for hypoxanthine of approximately 5 μM (Fig. 5). The hypoxanthine insertion reaction appeared to be irreversible, in that hypoxanthine, adenine,

guanine, cytosine, and uracil were incapable of exchanging the radiolabelled hypoxanthine out of the E. coli tRNA (data not shown).

DISCUSSION

Examination of the genetic code indicates which codons should permit inosine wobble with their tRNA counterparts. To insure fidelity of gene expression, only codons where uridine, cytidine, and adenosine in the third position all specify the same amino acid should be able to base-pair with inosine-containing tRNAs. These codon families include those for the amino acids alanine, arginine, glycine, isoleucine, leucine, proline, serine, threonine, and valine, and with the exception of glycine, these are exactly the same amino acids accommodated by tRNAs known to contain inosine in the anticodon wobble position (1). Therefore, the enzyme (or enzymes) responsible for the biosynthesis of inosine in the anticodon influences a broad spectrum of the genetic code.

The enzyme described in this investigation gives rise to inosine in tRNA by the covalent insertion of preformed hypoxanthine (Fig. 2). The only other tRNA modification enzyme known to insert a preformed base into tRNA is tRNA-guanine ribosyltransferase (4-7). Although that enzyme will catalyze a guanine for guanine exchange reaction in vitro (5), the normal function of the mammalian tRNA-guanine ribosyltransferase appears to be an exchange of guanine in the tRNA primary transcript for the modified base queuine (9). The nucleoside queuosine, like inosine, is found only in the first position of the anticodon of tRNAs (1).

That the queuosine and inosine modification reactions are catalyzed by different enzymes was established during this investigation. The queuine insertion enzyme and the hypoxanthine insertion enzyme were resolved by

DEAE-cellulose column chromatography, and the two enzymes were shown to have different substrate specificities *in vitro* (Fig. 1). In addition to the preferential use of hypoxanthine over guanine, *E. coli* tRNA was a preferred substrate for the putative tRNA-hypoxanthine ribosyltransferase *in vitro*. Unfractionated *E. coli* tRNA reportedly contains 60% less inosine than yeast tRNA (3), and this correlates quite well to the greater degree of hypoxanthine incorporation into *E. coli* tRNA than yeast tRNA (Fig. 4). In comparison, yeast tRNA is totally queuine deficient while *E. coli* tRNA is queuine modified, so the former is a much better substrate for the tRNA-guanine ribosyltransferase (9).

The need for a heterologous tRNA substrate *in vitro*, as demonstrated for the hypoxanthine insertion enzyme from human leukemia cells (Fig. 4), is as reported for other tRNA modification reactions (13). Since the homologous tRNAs are already fully modified *in situ* by the connate enzyme(s), they are poor substrates *in vitro*.

Preliminary kinetic analyses of the irreversible hypoxanthine incorporation into unfractionated *E. coli* tRNA yielded a K_m of approximately 5 μM for hypoxanthine using a crude cell extract from cultured human leukemia cells (Fig. 5). This value is comparable to the apparent K_m for guanine of 25 μM reported originally by Farkas and Singh (4) for the tRNA-guanine ribosyltransferase from rabbit erythrocytes. However, with purification of the guanine inserting enzyme, the affinity constant for guanine was found to be approximately 100-fold lower (5); apparently due to the removal of enzymes competing for the substrates of the reaction *in vitro*. Similar results might be obtained after purification of the extremely labile hypoxanthine insertion enzyme as well.

The identity of the base in the primary transcript being exchanged for hypoxanthine remains to be established. However, reports of microinjecting

cloned tRNA genes (14) or anticodon reconstructured tRNAs (15) into frog oocytes indicate that adenosine is being modified to inosine. In addition, with over 250 tRNAs sequenced to date (1), none contain adenosine in the first position of the anticodon, thereby further suggesting a hypoxanthine for adenine exchange. However, other base exchange possibilities are also being investigated with the rat liver and human leukemia cell tRNA modification enzymes.

Regardless of which base in the primary transcript is being replaced, inosine in the first position of the anticodon will greatly expand the wobble capability of the tRNA (2). Therefore, the newly discovered tRNA-hypoxanthine ribosyltransferase could be of importance in regulating codon recognition, and based on the number of codons potentially involved, this could have a major impact on gene expression.

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FOOTNOTES

[1] The abbreviations used are: Queuine, 7-(3,4-trans-4,5-cis-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine; queuosine, the ribonucleoside of queuine; HPLC, high-performance liquid chromatography; MEM, minimal essential medium.

FIGURE LEGENDS

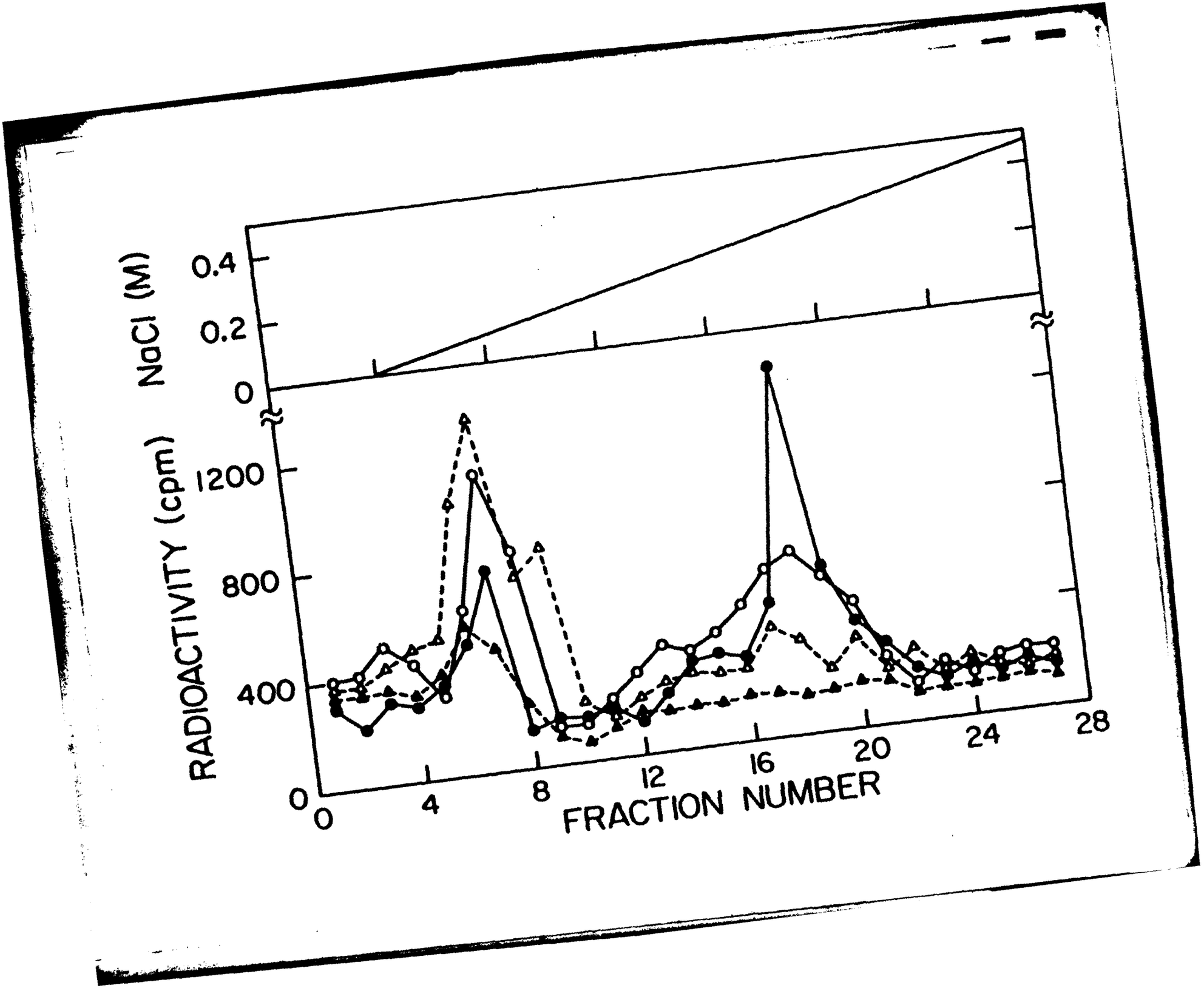
Fig. 1. Chromatography of rat liver tRNA ribosyltransferases on a DEAE-cellulose column. The chromatography and assay conditions were as described in EXPERIMENTAL PROCEDURES. The symbols indicate: E. coli tRNA and [8-^{3}H]hypoxanthine (●); yeast tRNA and [8-^{3}H]hypoxanthine (○); E. coli tRNA and [8-^{3}H]guanine (▲); and yeast tRNA and [8-^{3}H]guanine (△).

Fig. 2. HPLC elution profile of hydrolyzed E. coli tRNA radiolabelled with [8-^{3}H]hypoxanthine. Cultured HL-60 cells were used as a source of the enzyme. The enzyme assay, isolation and hydrolysis of the tRNA, and chromatography conditions were as described in EXPERIMENTAL PROCEDURES. The elution positions of authentic purine nucleosides and hypoxanthine are indicated.

Fig. 3. Relationship of reaction rate to enzyme protein concentration. Cultured CCRF-CEM cells were used as the source of the enzyme as described in EXPERIMENTAL PROCEDURES. The mean of triplicate assays is indicated at each protein concentration.

Fig. 4. Hypoxanthine insertion into tRNA. Cultured CCRF-CEM cells were used as a source of the enzyme. The assay conditions were as described in EXPERIMENTAL PROCEDURES with 0.03 mg protein from the leukemia cell extract per assay. The mean of triplicate assays is indicated for: E. coli tRNA (●), yeast tRNA (▲), and CCRF-CEM leukemia cell tRNA (■). CCRF-CEM tRNA was purified as described by Katze and Farkas (10).

Fig. 5. Lineweaver-Burk analysis for tRNA-hypoxanthine ribosyltransferase. Cultured CCRF-CEM cells were used as a source of the enzyme. The assay conditions were as described in EXPERIMENTAL PROCEDURES, with hypoxanthine concentrations from 0.75-15.75 μM. The mean of triplicate assays is indicated at each point.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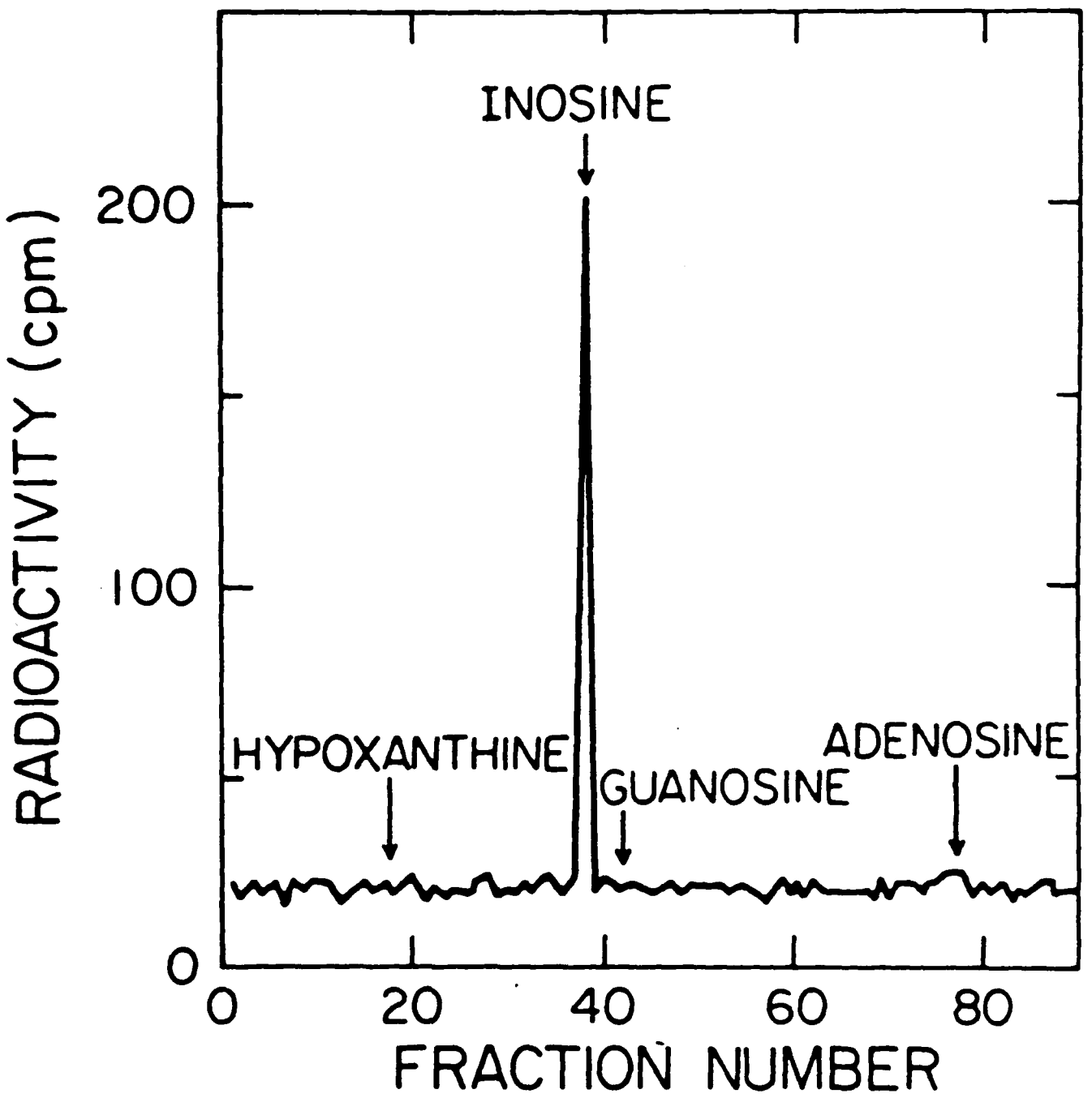
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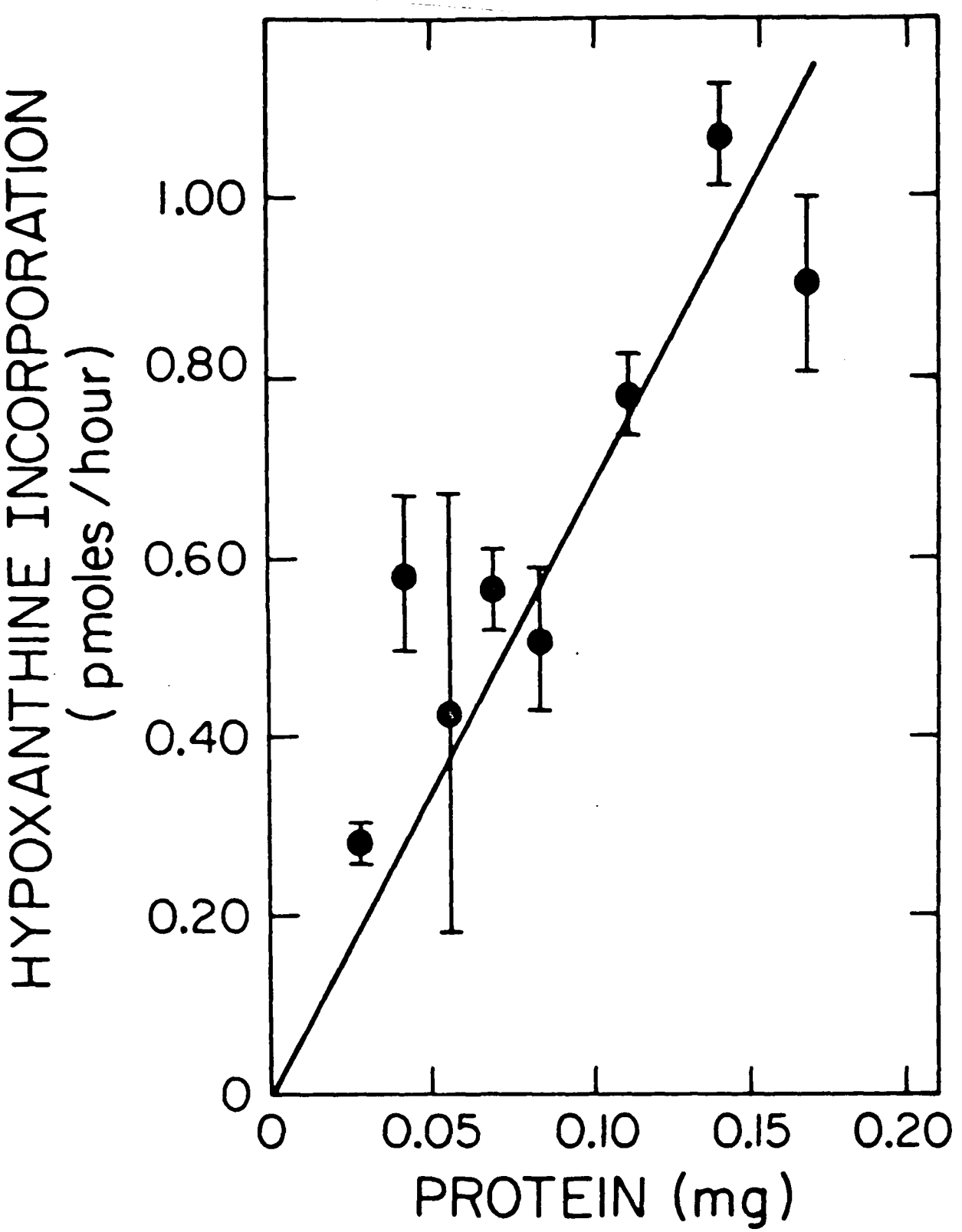
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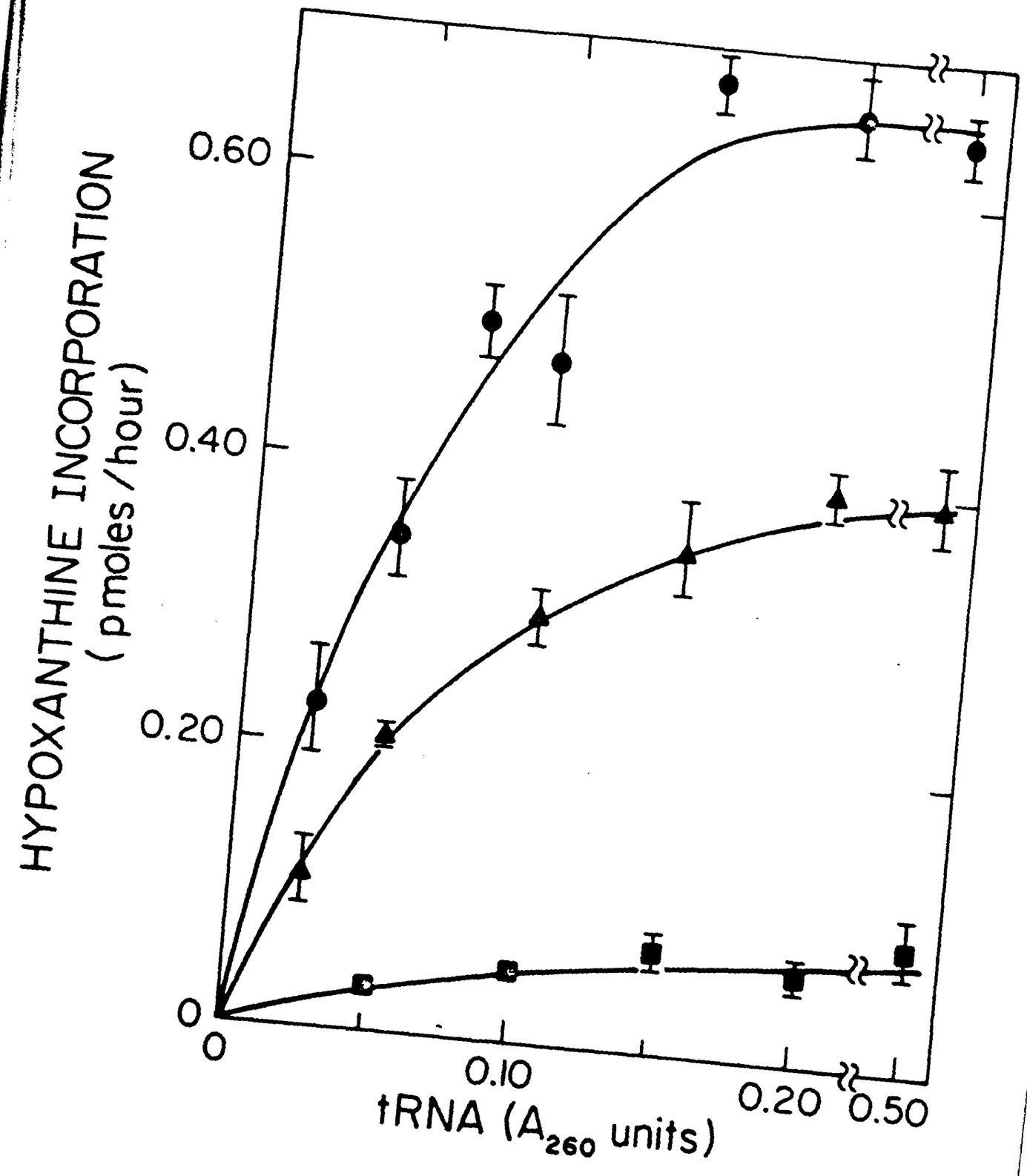
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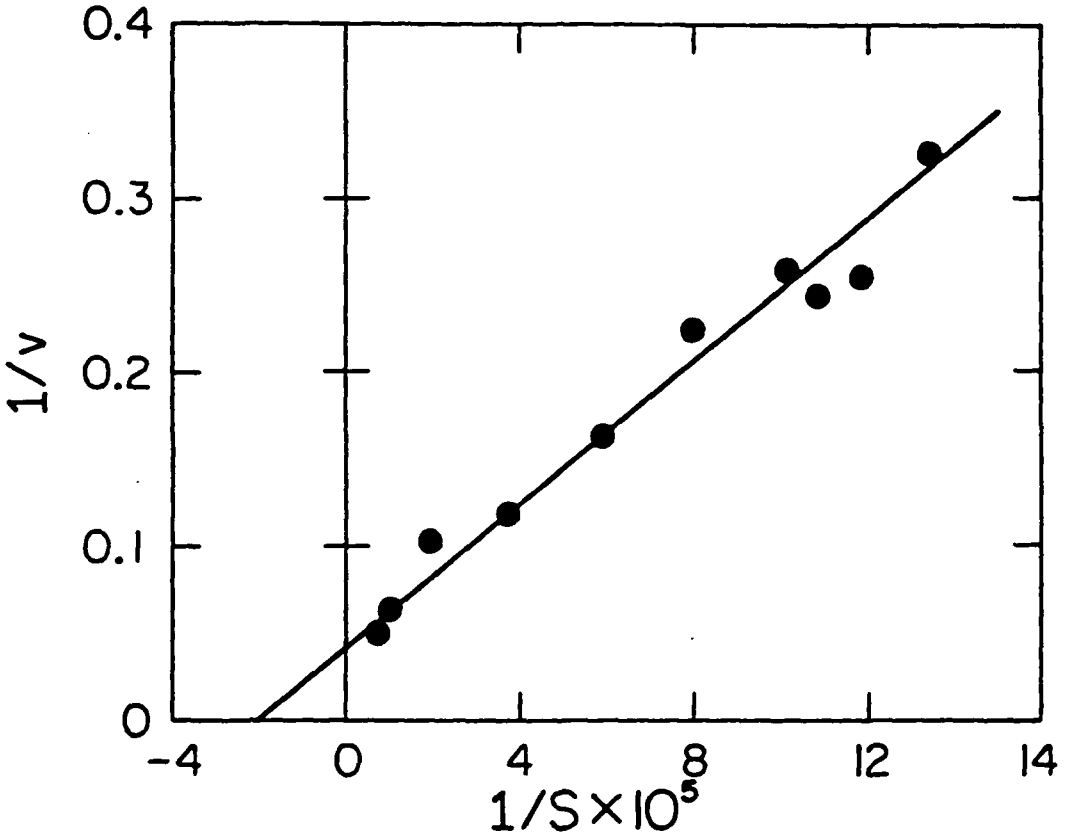
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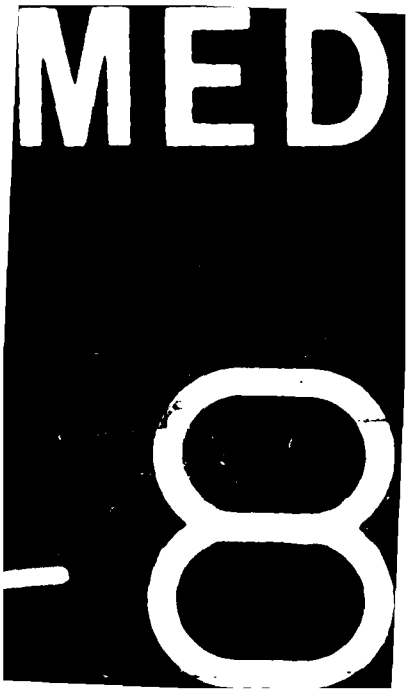
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